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(54) Title: GENES WHICH INFLUENCE <i>PICHIA</i> PROTEOLYTIC ACTIVITY, AND USES THEREFOR (57) Abstract The isolation and characterization of genes involved in proteolytic processing in species of the genus <i>Pichia</i> is described. The availability of such genes has enabled the generation of strains of <i>Pichia</i> that are deficient in proteolytic activity and useful as hosts for the expression of proteolytically sensitive recombinant products. The isolation and characterization of additional genes from species of the genus <i>Pichia</i> is also described, as well as uses therefor.		

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GENES WHICH INFLUENCE *PICHIA* PROTEOLYTIC
ACTIVITY, AND USES THEREFOR

This invention relates to recombinant DNA technology. In a particular aspect, the present invention relates to yeast strains produced employing recombinant techniques, and DNA encoding proteins involved in proteolytic processing, as well as auxotrophic marker proteins. In another aspect, the present invention relates to methods of producing recombinant products, especially recombinant products which are susceptible to proteolytic degradation.

10 BACKGROUND OF THE INVENTION

Strains of the genus *Pichia* have been developed as an efficient expression system for the production of recombinant products. Unfortunately, however, some protein products which are desirably produced by recombinant means (e.g., IGF-1, EGF, GRF, and the like) are susceptible to degradation by proteases produced by the host organism. In such cases, even if high levels of the desired product are expressed, reduced product recoveries are sometimes realized due to degradation of the product in the presence of certain of the host strain's proteolytic enzymes. Product recovery is further complicated by the presence of various proteolysis degradation products.

It would be desirable, in view of the excellent performance of the *Pichia*-based expression system for the production of many recombinant products, to reduce or eliminate certain proteolytic activities of *Pichia*. This would reduce the likelihood of degradation of protease-sensitive products when produced in recombinant

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Pichia hosts. Reduced likelihood of degradation would result in an enhanced ability to express and recover such products in substantially intact form.

Various techniques can be applied in an effort to
5 reduce or eliminate the problem of proteolytic
degradation of recombinantly produced products. For
example, one could modify the conditions under which
recombinant strains are grown so as to inhibit protease
activity. This could be accomplished, for example, by
10 adjusting the pH of the medium sufficiently to inhibit
the action of various proteases. This approach, however,
may affect the ability of the host organism to express
certain recombinant products (as well as the stability of
the resulting product, once expressed). Moreover, this
15 approach is limited only to its effect on extracellular
proteolysis.

Alternatively, one could attempt to modify or
eliminate some or all of the host organism's processing
enzymes which are responsible for the proteolytic
20 activity which degrades recombinantly produced,
proteolytically sensitive products. Proteolytic
processes in eukaryotic organisms are, however, quite
complicated and involved. Thus, it is not possible to
predict if elimination and/or modification of one or more
25 of the enzyme(s) that are involved in proteolytic
processing pathways will have an impact on the viability
of the host cells, and/or the stability of the
recombinantly produced products.

Some of the proteolytic activities of the yeast
30 *Saccharomyces cerevisiae* have been characterized.
Proteinase A, for example, is encoded by the *S.*
cerevisiae *PEP4* gene. Proteinase A is a vacuolar,
aspartyl protease capable of self-activation, as well as
subsequent activation of additional vacuolar proteases,
35 such as carboxypeptidase Y, and proteinase B. Although

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carboxypeptidase Y appears to be completely inactive prior to proteinase A-mediated proteolytic processing of the enzyme, proteinase B (encoded by the PRB-1 gene of *S. cerevisiae*) reportedly is approximately 50% bioactive in its precursor form, the form that exists prior to proteinase A-mediated processing of the enzyme.

S. cerevisiae and filamentous fungi deficient in proteolytic activity have been used for the recombinant expression of heterologous peptides. These organisms, however, differ substantially from the methylotrophic yeast, *Pichia*. There are numerous metabolic and physiological differences between *Saccharomyces*, *Aspergillus*, and *Pichia*, so that the proteolytic processing systems of these various organisms are not necessarily similar. Indeed, very little is presently known regarding the types of proteolytic activities present in *Pichia*.

In addition, unlike *Saccharomyces* or *Aspergillus*, *Pichia* cells used for the recombinant expression of heterologous peptides are typically grown to high cell density, which has been made possible, at least in part, by selection of strains which minimize the occurrence of foaming during the fermentation process. Selection of such cells is accomplished by selecting for cells which produce large amounts of endo- and exo-proteases, which reduce foaming by reducing the size of proteins secreted into the medium. Furthermore, while growth at high cell density the production of heterologous peptides in high yields, growth at high cell density also provides for a relatively high level of vacuolar proteases in the fermentation medium. The high cell density is accompanied by the release of substantial quantities of cellular material into the media, including vacuolar proteases, since ~1% of cells typically undergo lysis during yeast fermentation. Therefore, during the

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production of heterologous peptides in a high cell density process, some of the secreted, heterologous peptides produced by *Pichia* could be subjected to substantial proteolysis.

5 Therefore, it is an object herein, to provide protease deficient strains of *Pichia* and to provide means for generating such strains. It is also an object to use the protease deficient strains for expression of heterologous proteins.

10 **SUMMARY OF THE INVENTION**

 In accordance with the present invention, we have isolated and characterized genes involved in proteolytic processes of species of the genus *Pichia*. The availability of such genes provides a means to generate
15 strains of *Pichia* which are deficient in proteolytic activity and which are useful as hosts for the expression of proteolytically sensitive products.

 The strains of *Pichia* which have been modified so as to be defective in proteolytic activity, compared to
20 wild-type *Pichia* cells, are excellent hosts for the expression of recombinant constructs encoding proteolytically sensitive products. The advantage of high levels of recombinant product expression using the *Pichia* expression system, coupled with the low level of
25 proteolytic activity in the protease-deficient host cells provided herein, provides a highly efficient expression system for the production of proteolytically sensitive products.

 In accordance with another embodiment, a gene that
30 encodes the *Pichia* orotidine-5'-phosphate decarboxylase protein (the *URA3* gene) is provided. The availability of this gene, in combination with strains of *Pichia* which are *Ura⁻*, provides a selection system for use in producing recombinant strains of *Pichia* which are deficient in
35 proteolytic activity. Such *Ura⁻* strains are also useful

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as hosts for transformation with recombinant DNA constructs, which are then used for the recombinant expression of a variety of heterologous products.

BRIEF DESCRIPTION OF THE FIGURES

5 Figure 1 is a restriction map of a *Pichia pastoris* gene which influences the carboxypeptidase Y activity of *Pichia*.

Figure 2 is a restriction map of plasmid pEP202.

Figure 3 is a restriction map of plasmid pEP205.

10 Figure 4 is a restriction map of plasmid pEP301.

Figure 5 is a restriction map of plasmid pDR401.

Figure 6 is a restriction map of plasmid pPU201.

Figure 7 is a restriction map of plasmid pPU202.

Figure 8 is a restriction map of plasmid pPU203.

15 Figure 9 is a restriction map of plasmid pPU205.

Figure 10 is a restriction map of plasmid pPU206.

Figure 11 is a restriction map of plasmid pDR421.

20 Figure 12 is a restriction map of the *Pichia pastoris* orotidine-5'-phosphate decarboxylase gene (i.e., the *URA3* gene).

Figure 13 summarizes the steps employed in the construction of pDR601 and pDR602.

Figure 14 is a restriction map of plasmid pDR601.

Figure 15 is a restriction map of plasmid pDR602.

25 Figure 16 is a restriction map of plasmid pDL521.

Figure 17 is a restriction map of a portion of the *Pichia pastoris* proteinase B gene.

Figure 18 is a restriction map of plasmid pDR911.

DETAILED DESCRIPTION OF THE INVENTION

30 In accordance with the present invention, there is provided an isolated DNA fragment obtained from a strain of the genus *Pichia* which comprises a gene encoding a protein which, directly or indirectly, influences the proteolytic activity of said strain.

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In accordance with another embodiment of the present invention, there is provided a method of producing modified strain(s) of the genus *Pichia* which are deficient in proteolytic activity, relative to host strain(s) of the same species which are not so modified, said method comprising:

contacting said host strain(s) with a modified form of the above-described gene, wherein said modification renders the gene incapable of producing functional product, or alters the ability of the gene product to influence proteolytic activity, wherein said contacting is carried out under conditions suitable for the site-directed integration of said modified form of the above-described gene into the genome of said host strain(s), wherein said site-directed integration occurs at the specific locus of said gene which encodes said protein which influences proteolytic activity.

In accordance with yet another embodiment of the present invention, there are provided strains of the genus *Pichia* which are deficient in proteolytic activity. Such strains can be produced in a variety of ways, with the above-described method being the presently preferred way of producing such strains.

In accordance with still another embodiment of the present invention, there is provided a method for the expression of proteolytically sensitive recombinant product(s), said method comprising expressing said proteolytically sensitive product(s) in the above-described *Pichia* cells which are deficient in proteolytic activity.

In accordance with a further embodiment of the present invention, there is provided an isolated DNA fragment obtained from a species of the genus *Pichia* which comprises the orotidine-5'-phosphate decarboxylase gene.

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In accordance with a still further embodiment of the present invention, there is provided a yeast cell of the genus *Pichia* as a host capable of being transformed with recombinant DNA material, wherein said host is defective
5 in the orotidine-5'-phosphate decarboxylase gene.

As employed herein, the term "proteolytic activity" refers to any one or more of the enzyme activities displayed by enzymes involved in the proteolytic pathway. Proteolytic activities include proteinase A activity,
10 proteinase B activity, carboxypeptidase Y activity, carboxypeptidase S activity, aminopeptidase C activity, dipeptidyl aminopeptidase activity, proteinase D activity, proteinase E activity, and the like.

As used herein, a gene encoding a protein which,
15 directly or indirectly, influences the proteolytic activity of a yeast strain, includes genes that encode proteinases or that encode proteins that act on proteinases. As used herein, a protein that acts on a protein refer to proteins that alter or modulate the
20 activity of a proteinase. Thus, for example, a protein that directly influences proteolytic activity is a protein that encodes a proteinase, and a protein that indirectly influences proteolytic activity is a protein that activates or increases the activity of a protein by
25 proteolytic processing. *Saccharomyces cerevisiae* proteinase is an example of a protein that directly and indirectly influences the proteolytic activity of *Saccharomyces cerevisiae*.

In accordance with one embodiment of the present
30 invention, the *Pichia* gene that encodes a protein which, directly or indirectly, influences at least the carboxypeptidase Y activity of strains of the genus *Pichia* has been identified and isolated from a species of the genus *Pichia*. This gene is referred to herein, for
35 convenience, as the *Pichia* PEP4 gene, based on the

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existence of some similarity between this gene and the *S. cerevisiae* PEP4 gene. It should be recognized, however, that the nucleotide sequences of the *Pichia* gene and the *Saccharomyces* gene differ substantially. The *Pichia* PEP4 gene is characterized by the restriction map set forth in Figure 1 of the drawings. A fragment containing sequences encoding this gene can be readily obtained for easy handling from a variety of sources. One such source is the approximately 10.6 kbp EcoRI fragment of plasmid pEP202 (see Figure 2), or alternatively, the approximately 2.7 kbp EcoRI-SacI fragment of plasmid pEP301 (see Figure 4).

DNA encoding the proteinase A gene is also provided. The proteinase A gene of the present invention can be further characterized by reference to the amino acid sequence set forth in Sequence ID No. 2. DNA having any nucleic acid sequence which encodes substantially the same amino acid sequence as set forth in Sequence ID No. 2 or that has sufficient homology to be useful for disruption of homologous genes can be employed in the practice of the present invention. An exemplary nucleic acid sequence which encodes the above-described amino acid sequence is set forth in Sequence ID No. 1.

The *Pichia* gene that encodes a protein which, directly or indirectly, influences the proteolytic activity of strains of the genus *Pichia* can be modified in a variety of ways, so as to render the gene incapable of producing functional product, or so as to alter the ability of the gene product to influence the proteolytic activity of said *Pichia* strain(s). Those of skill in the art recognize that there are many methods for the modification of the above-described gene. For example, the coding sequence can be mutated to modify the amino acid sequence of the protein encoded by the gene. Alternatively, various portions of the coding sequence

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can be deleted from the gene. The deletion need only be sufficient to render the expressed product (if it is still capable of being expressed) non-functional. Thus, a deletion of even one nucleotide, by throwing the remaining coding sequence out of reading frame, can render a product, if still capable of expression, non-functional. Of course, larger deletions can result in a complete lack of expression of product, or can cause a substantially modified product to be expressed, and such a product is likely to have very different proteolytic properties, if any, relative to product produced by intact gene. As yet another alternative, additional sequences can be inserted into the coding sequence to disrupt the reading frame of the gene of interest, which would cause a dramatically altered product to be expressed, or a complete lack of expression of the product.

A particularly convenient method for the modification of the *Pichia* gene that encodes a protein which, directly or indirectly, influences the proteolytic activity of strains of the genus *Pichia* is to insert an auxotrophic marker gene into said *Pichia* gene, thereby disrupting the *Pichia* gene. Such auxotrophic marker genes can be selected from the *Pichia* or *Saccharomyces* *HIS4* gene, the *Pichia* or *Saccharomyces* *ARG4* genes, the *Pichia* or *Saccharomyces* *URA3* genes, and the like.

Strains of *Pichia* deficient in proteolytic activity can be prepared in a variety of ways. The presently preferred method involves modifying, in a suitable host, genes of the present invention (which genes, in their unmodified form, encode a product which, directly or indirectly, affects the proteolytic activity of strains of the genus *Pichia*). Alternatively, host strains can be subjected to random (i.e., non-selective) mutagenesis,

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then screened to select for mutants which are deficient in proteolytic activity.

When proteolytically deficient strains are produced by modifying the gene of the invention in a host, such
5 modifying is carried out, for example, by introducing a modified gene under transformation conditions suitable for the site-directed integration of the modified gene into the genome of the host at the specific locus of such gene which encodes a protein which influences proteolytic
10 activity (i.e., the target gene). Integration will replace or alter the host's endogenous gene. A convenient means to introduce the modified gene into the target locus of a yeast host is to include the modified gene in a linear DNA fragment having ends homologous to
15 two separate portions of the intact gene within the host. This will direct, upon transformation, homologous recombination occur at the specific locus of the gene whose expression product influences proteolytic activity.

When *Pichia* strains deficient in proteolytic
20 activity are prepared by the preferred method described above (i.e., by introducing a modified gene of the invention into a suitable host by site-directed integration at the specific locus of the gene whose expression product influences proteolytic activity,
25 thereby replacing all or a portion of the endogenous gene with all or a portion of the modified gene), the endogenous gene is said to be disrupted.

As used herein, the term gene "disruption" refers to any manipulation of the target locus that ultimately
30 results in the presence of a gene that does not yield a functional product, or that yields a product with altered function. Disruption can, therefore, result from the presence of added sequence (e.g., by the introduction of auxotrophic marker, or by the introduction of any
35 sequence which causes a shift in the reading frame), the

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loss of nucleotides from the target gene (e.g., by deletion), or other mutations of the target gene. For the preferred method of preparing *Pichia* strains deficient in proteolytic activity, gene disruption is

5 achieved by gene addition, gene replacement, or a combination of addition and replacement referred to herein as "pop-in-pop-out". In gene replacement, the endogenous target gene is physically removed from the target locus, and replaced with the modified gene. This

10 is accomplished by transforming the host with a linear fragment having ends which are homologous to the 5' and 3' ends of the target gene, respectively. Gene addition involves adding the transforming DNA to the endogenous target gene. Depending on the manner in which the

15 modified gene of the transforming DNA was altered, gene addition can result in the presence of either two non-functional copies of the target gene, or one functional and one non-functional copy of the target gene. Each of the two copies consists of a portion of the endogenous

20 gene, and a portion of the transforming DNA. If a functional copy of the target gene remains after gene addition, it can then be removed by homologous recombination between the two copies of the target gene. The combination process of gene addition followed by

25 homologous recombination constitutes the pop-in-pop-out process.

Methods of transforming yeast of the genus *Pichia*, as well as methods applicable for culturing such yeast cells, are known generally in the art. Constructs

30 containing the above-described modified gene are transformed into *Pichia* cells either by the spheroplast technique, described by Cregg et al., in Mol. Cell. Biol. 5:3376 (1985) and U.S. 4,879,231, or by the whole-cell lithium chloride yeast transformation system [Ito et al.,

35 Agric. Biol. Chem. 48:341 (1984)], with modification

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necessary for adaptation to *Pichia* [See European Patent Application No. 312,934; also available as U.S. Pat. No. 4,929,535]. The whole-cell lithium chloride method is frequently more convenient in that it does not require
5 the generation and maintenance of spheroplasts. For the purpose of the present invention, the spheroplast method is preferred because the spheroplast method is generally a more efficient means of transformation.

Those of skill in the art recognize that host *Pichia*
10 strains for transformation with the above-described modified gene can be wild-type *Pichia* cells, which upon transformation with a defective gene from the proteolytic pathway, could be screened for reduced proteolytic activity. The host strains employed can have one or more
15 defects therein, to assist in the identification and selection of desired transformants.

Preferred hosts employed for transformation with a modified form of the gene which encodes a protein which, directly or indirectly, influences the proteolytic
20 activity of strains of *Pichia*, is a strain which is defective in at least one auxotrophic marker gene. The use of such host organisms is preferred because simultaneous transformation of such a host with the modified form of the invention gene and an auxotrophic
25 marker gene enables rapid selection of strains which have incorporated the transforming DNA, and thus, should have a disrupted form of the gene which encodes a protein which directly or indirectly influences the proteolytic activity of the host.

30 Exemplary auxotrophic marker genes useful in the practice of the present invention (i.e., marker genes that are defective in the preferred host strains employed herein) include the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the
35 orotidine-5'-phosphate decarboxylase gene, and the like.

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When employing such host strains in the transformation of *Pichia*, the above-described modified gene, included on a linear DNA fragment, is preferably associated with an intact form of the auxotrophic marker gene for which the host strain is defective, e.g., the auxotrophic marker gene either is contained within the modified gene, or is located 5' or 3' of the modified gene on the transforming linear DNA fragment. Exemplary host strains contemplated for use in the practice of the present invention include the *HIS4*-defective *Pichia* strain, GS115 (ATCC 20864), the *ARG4*-defective *Pichia* strain, GS190, the *HIS4/URA3*-defective *Pichia* strain, GS4-2, the *HIS4/ARG4*-defective *Pichia* strain PPF1 (NRRL Y-18017; see U.S. 4,812,405), and the like. An exemplary fragment of DNA which contains the above-described modified gene having inserted therein a functional gene encoding histidinol dehydrogenase can be obtained from the approximately 5.3 kbp *SacI*-*EcoRI* fragment of plasmid pDR401. Another exemplary fragment of DNA which contains a modified form of the above-described gene (located 5' of a functional gene encoding orotidine-5'-phosphate decarboxylase) can be obtained from the approximately 5.0 kbp *BglIII* fragment of plasmid pDR421.

A particularly advantageous application of the *Pichia* strains that are deficient in proteolytic activity is for the expression of proteolytically sensitive recombinant products, such as, for example, epidermal growth factor (EGF), growth hormone releasing factor (GRF), insulin-like growth factor-1 (IGF-1), and the like. When expressed in recombinant *Pichia* strains, which are deficient in proteolytic activity, the resulting recombinant product is subjected to a reduced level of proteolytic activity, due to modifications in the proteolysis apparatus of the host organism.

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Proteolytically deficient *Pichia* expression systems for the production of proteolytically sensitive products can be generated in a variety of ways. For example, *Pichia* host strains can be rendered proteolytically deficient, as described hereinabove, and then further transformed with DNA encoding a heterologous protein of interest (especially a proteolytically sensitive protein). Alternatively, a recombinant *Pichia* strain already bearing DNA encoding a heterologous protein of interest can thereafter be rendered proteolytically deficient, for example, as described hereinabove. As yet another alternative, a *Pichia* strain could be co-transformed with the above described modified gene and a DNA encoding a heterologous, proteolytically sensitive protein of interest.

The use of strains of the genus *Pichia* as host strains in the recombinant expression of peptide products has previously been described in great detail. The presently preferred yeast species for use in the practice of the present invention is *Pichia pastoris*, a known industrial yeast strain that is capable of efficiently utilizing methanol as the sole carbon and energy source.

There are a number of methanol-responsive genes in methylotrophic yeast, the expression of each being controlled by methanol-responsive regulatory regions (also referred to as promoters). Any of such methanol-responsive promoters are suitable for use in the practice of the present invention. Examples of specific regulatory regions include the promoter for the primary alcohol oxidase gene from *Pichia pastoris* AOX1, the promoter for the secondary alcohol oxidase gene from *P. pastoris* AOX2 (*P. pastoris* is known to contain two functional alcohol oxidase genes: alcohol oxidase I (AOX1) and alcohol oxidase II (AOX2); the coding portions

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of the two AOX genes are closely homologous at both the DNA and the predicted amino acid sequence levels and share common restriction sites; the proteins expressed from the two genes have similar enzymatic properties but
5 the promoter of the AOX1 gene is more efficient and gene products are frequently more highly expressed therefrom), the promoter for the dihydroxyacetone synthase gene from *P. pastoris* (DAS), the promoter for the P40 gene from *P. pastoris*, the promoter for the catalase gene from *P. pastoris*, the promoter for the formaldehyde dehydrogenase
10 gene from *P. pastoris*, the promoter for the formate dehydrogenase gene from *P. pastoris*, and the like.

The presently preferred promoter region for regulating expression of a gene encoding a
15 proteolytically sensitive product, in *P. pastoris* hosts, is the promoter of the methanol-regulated primary alcohol oxidase gene of *P. pastoris*. The AOX1 gene, including its promoter, has been isolated and thoroughly characterized; see Ellis et al., Mol. Cell. Biol. 5: 1111
20 (1985) and U.S. Patent No. 4,855,231.

The presently preferred expression cassette used in transforming *Pichia* cells for the generation of recombinant protein-expressing strains comprises, in the reading frame direction of transcription, the following
25 DNA sequences:

- (i) a promoter region of a methanol-responsive gene of a methylotrophic yeast,
- (ii) a DNA sequence encoding a polypeptide consisting essentially of:
30 (a) an optional secretion signal sequence, and
(b) a heterologous protein of interest; and
- (iii) a transcription terminator functional in a methylotrophic yeast;

wherein said DNA sequences are operationally associated
35 with one another for transcription of the sequences

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encoding said polypeptide. DNA sequences encoding a secretion signal sequence which are optionally contained in expression vectors used in the practice of the present invention include the DNA encoding the native secretion
5 signal sequence associated with the proteolytically sensitive product, the DNA encoding the *S. cerevisiae* α -mating factor (α MF) leader sequence, (including a DNA sequence encoding the processing site, lys-arg), and signal sequences that function as such in methylotrophic
10 yeast cells, such as the bovine lysozyme C signal sequence.

The transcription terminator functional in a methylotrophic yeast used in accordance with the present invention has either (a) a subsegment which provides a
15 polyadenylation signal and polyadenylation site in the transcript, and/or (b) a subsegment which provides a transcription termination signal for transcription from the promoter used in the expression cassette. The term
"expression cassette" as used herein, and throughout the
20 specification and claims, refers to a DNA sequence which includes sequences functional for the expression process. The entire transcription terminator is taken from a protein-encoding gene, which may be the same or different from the gene which is the source of the promoter.

25 In the DNA constructs of the present invention, used to transform hosts for recombinant expression of proteolytically sensitive products, the segments of the expression cassette(s) are said to be "operationally associated" with one another. The DNA sequence encoding
30 proteolytically sensitive products is positioned and oriented functionally with respect to the promoter, the secretion signal sequence, if employed, and the transcription terminator. Thus, the polypeptide-encoding segment is transcribed, under regulation of the promoter
35 region, into a transcript capable of providing, upon

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translation, the desired polypeptide. Appropriate reading frame positioning and orientation of the various segments of the expression cassette are within the knowledge of persons of ordinary skill in the art;

5 further details are given in the Examples.

For the practice of the present invention it is preferred that hosts for the recombinant expression of proteolytically sensitive products be transformed with multiple copies of the above-described expression
10 cassettes contained on one DNA fragment, preferably in a head-to-tail orientation.

In addition, when DNA constructs according to the invention are used to transform hosts for the recombinant expression of proteolytically sensitive products by
15 site-directed integration, the expression cassette-containing construct is a linear DNA fragment that is directed to the desired locus of the host to effect integration of the DNA fragment therein. One-step gene integrations are usually successful if the DNA to be
20 introduced has as little as 0.2 kb homology with the fragment locus of the target gene; it is however, preferable to maximize the degree of homology for efficiency.

The DNA constructs used according to the invention
25 to transform hosts for the recombinant expression of proteolytically sensitive products optionally further comprise a selectable marker gene, in addition to one or more expression cassettes. For this purpose, any selectable marker gene functional in methylotrophic yeast
30 may be employed, i.e., any gene which confers a phenotype upon methylotrophic yeast cells, thereby allowing them to be identified and selectively grown from among a vast majority of untransformed cells. Suitable selectable marker genes include, for example, selectable marker
35 systems composed of an auxotrophic mutant *P. pastoris*

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host strain and a wild-type biosynthetic gene which complements the host's defect. For transformation of *HIS4*⁻ *P. pastoris* strains, for example, the *S. cerevisiae* or *P. pastoris HIS4* gene may be employed, or for
5 transformation of *ARG4*⁻ mutant *P. pastoris* strains, the *S. cerevisiae ARG4* gene or the *P. pastoris ARG4* gene may be employed, or for transformation of *URA3*⁻ mutant *P. pastoris* strains, the *S. cerevisiae URA3* gene or the *P. pastoris URA3* gene may be employed.

10 In addition, DNA constructs used to transform hosts for the recombinant expression of proteolytically sensitive products according to this aspect of the invention optionally further comprise selectable marker genes which are functional in bacteria. Thus, any gene
15 can be used which confers a phenotype on bacteria that allows transformed bacterial cells to be identified and selectively grown from among a vast majority of untransformed cells. This additional selectable marker enables DNA of the invention to be transformed into
20 bacteria such as *E. coli* for amplification. Suitable selectable marker genes include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r), and the like.

When it is contemplated to pass DNA of the invention
25 through bacterial cells, it is desirable to include in the DNA construct a bacterial origin of replication, to ensure the maintenance of the invention DNA from generation to generation of the bacteria. Exemplary bacterial origins of replication include the *fl*-ori, *col*isin, *col* E1, and the like.
30

The term "expression vector", as employed herein, is intended to include vectors capable of expressing DNA sequences contained therein, where such sequences are in operational association with other sequences capable of
35 effecting their expression, i.e., promoter sequences. In

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general, expression vectors usually used in recombinant DNA technology are often in the form of "plasmids", i.e., circular, double-stranded DNA loops, which in their vector form are not bound to the chromosome. In the present specification the terms "vector" and "plasmid" are used interchangeably. However, the invention is intended to include other forms of expression vectors as well, which function equivalently.

Methods of transforming yeast of the genus *Pichia*, as well as methods applicable for culturing such yeast cells, are known generally in the art.

According to the invention, constructs containing the above-described modified gene and/or expression cassettes encoding the production of heterologous, proteolytically sensitive products are transformed into *Pichia* cells either by the spheroplast technique, or by the whole-cell lithium chloride yeast transformation system, as described above.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors in either batch or continuous mode. For the large-scale production of recombinant DNA-based products in methylotrophic yeast, a three-stage, high cell-density fermentation system is the presently preferred fermentation protocol employed. In the first, or growth stage, expression hosts are cultured in defined minimal medium with an excess of a non-inducing carbon source (e.g., glycerol). When grown on such carbon sources, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. It is presently preferred, during this growth stage, that the pH of the medium be maintained at about 5, because the *P. pastoris* cells generally prefer a pH of about 5 for optimal growth. Next, a short period of non-inducing carbon source

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limitation growth is allowed to further increase cell mass and derepress the methanol-responsive promoter. The pH of the medium during this limitation growth period is maintained at an appropriate pH value (the actual pH employed is a function of the particular host strain used for expression and the specific product being expressed).

Subsequent to the period of growth under limiting conditions, methanol is added in the fermentor either on a continuous basis, with concurrent removal of product via the broth; or on a batch-wise basis wherein methanol is added at such a rate that the methanol content of the broth is maintained at a low level (referred to herein as "methanol excess fed-batch mode"). The addition of methanol induces the expression of the gene driven by a methanol-responsive promoter. This third stage is referred to as the production stage, because it is at this stage that the majority of the recombinant product is expressed. The pH of the medium during the production stage is maintained at an appropriate pH value (the actual pH employed is a function of the particular host strain used for expression and the specific product being expressed).

The term "culture" means a propagation of cells in a medium conducive to their growth, and all sub-cultures thereof. The term "subculture" refers to a culture of cells grown from cells of another culture (source culture), or any subculture of the source culture, regardless of the number of subculturing steps that have been performed between the subculture of interest and the source culture.

According to a preferred embodiment of the present invention, the heterologous protein expression system used for the production of proteolytically sensitive products utilizes the promoter derived from the methanol-regulated AOX1 gene of *P. pastoris*, which is very

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efficient and tightly regulated. This gene can be the source of the transcription terminator as well. The presently preferred expression cassette comprises, operationally associated with one another, the P.

5 *pastoris* AOX1 promoter, optional DNA encoding a secretion signal sequence, a DNA sequence encoding a proteolytically sensitive product (e.g., mature IGF-1, EGF, GRF, and the like), and a transcription terminator derived from the *P. pastoris* AOX1 gene. Preferably, two
10 or more of such expression cassettes are contained on one DNA fragment, in head-to-tail orientation, to yield multiple expression cassettes on a single contiguous DNA fragment.

The presently preferred host cells to be transformed
15 with multiple expression cassettes are *P. pastoris* cells having at least one mutation that can be complemented with a marker gene present on a transforming DNA fragment. Preferably *HIS4*⁻ (GS115) or *ARG4*⁻ (GS190) single auxotrophic mutant *P. pastoris* strains are employed, or
20 *HIS4*⁻/*URA3*⁻ (GS4-2) or *HIS4*⁻/*ARG4*⁻ (PPF1) double auxotrophic mutant *P. pastoris* strains are employed.

The fragment containing one or more expression cassette(s) is inserted into a plasmid containing a marker gene complementing a metabolic defect in the host,
25 and optionally containing additional sequences such as bacterial marker genes, yeast DNA sequences which direct vector integration, and the like.

In accordance with a specific embodiment of the present invention, there is provided an isolated DNA
30 fragment obtained from a species of the genus *Pichia* which comprises the orotidine-5'-phosphate decarboxylase gene. The orotidine-5'-phosphate decarboxylase gene is frequently referred to as *URA3*. It can be used, for example, to complement *URA3*-deficient strains. Another
35 use for the novel gene is the ability to target DNA into

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a specific locus of the *Pichia* genome (i.e., into the *URA3* locus). This novel gene can be characterized by reference to the restriction map shown in Figure 12. Alternatively, this novel gene can be characterized as
5 encoding a protein having substantially the same amino acid sequence as set forth in Sequence ID No. 4. While those of skill in the art recognize that the above-referenced amino acid sequence can be encoded by a variety of nucleotide sequences, a presently preferred
10 nucleotide sequence encoding the above-referenced amino acid sequence is substantially the same as that set forth in Sequence ID No. 3.

In accordance with another specific embodiment of the present invention, there are provided yeast cells of
15 the genus *Pichia* as a host capable of being transformed with recombinant DNA material, wherein the host is defective in the orotidine-5'-phosphate decarboxylase gene. Host strains defective in the *URA3* gene can be used for transformation with DNA containing an intact
20 form of the *URA3* gene, thereby enabling a ready determination of whether the desired transformation event has occurred (by return of successfully transformed cells to uracil prototrophy).

The combination of *URA3* *Pichia* strains and
25 the *Pichia* orotidine-5'-phosphate decarboxylase marker gene provides a particularly useful selection system for use in producing recombinant strains of *Pichia* deficient in proteolytic activity. Such a selection system is referred to herein as a "bidirectional
30 selection process". This selection system for the generation of *Pichia* strains which are deficient in proteolytic activity uses a "pop-in-pop-out" gene disruption technology in which a DNA fragment containing a defective gene is added to the genome
35 of a host organism, with subsequent removal of

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portions of the DNA fragment and endogenous sequences from the host through homologous recombination between the endogenous target gene sequence and the integrated vector sequence. Initially, transformants are selected for incorporation of the disruption vector which contains a marker gene such as *URA3* (i.e., the "pop-in" step). Next, the selected transformants must be screened to identify strains in which a recombination event between endogenous gene sequences and integrated vector sequences has occurred and has thereby excised portions of the vector, including the marker gene, and endogenous sequences of the host (i.e., the "pop-out" step). A double selection system based on the *URA3* gene and *URA3*⁻ hosts provides for the sequential identification of the desired strains.

This type of gene disruption is typically conducted in *Ura*⁻ strains, which can be identified by resistance to 5-fluoro-orotic acid (5-FOA). Disruption vectors contain a defective copy of the target gene to be disrupted and a functional *URA3* gene. Integration of the disruption vector into the genome of the *Ura*⁻ host cells generates *Ura*⁺ transformants containing one functional target gene and one non-functional (i.e., defective) target gene. *Ura*⁺ transformants are easily identified by their ability to grow in the absence of uracil.

In order to isolate strains in which a recombination event has resulted in the elimination of the functional target gene, leaving only a defective gene, the *Ura*⁺ transformants are screened for restoration of 5-FOA resistance resulting from the loss ("pop-out") of the *URA3* gene which accompanies recombination. The regeneration of the *URA3* genotype enables repetition of the "pop-in-pop-out" process for the subsequent disruption of other genes in the genome. To use this selection system for the generation of *Pichia* strains

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which are deficient in proteolytic activity, a *URA3⁻* host is transformed with a DNA construct containing a modified form of a gene encoding a protein involved in the *Pichia* proteolytic pathway, and the *URA3* gene. Site-directed
5 integration of the transforming DNA by gene addition (i.e., "pop-in") yields one functional and one non-functional gene at the locus of the gene which directly or indirectly influences proteolytic activity, as well as an intact *URA3* gene. Strains which incorporate the *URA3*
10 gene are identified by positive selection (using techniques well known to those of skill in the art, e.g., by growing the strains on minimal media lacking uracil and selecting those strains capable of growth on such media). The configuration of the functional, non-
15 functional and *URA3* genes at the locus of the gene which encodes a protein which influences proteolytic activity enables recombination to occur between the functional and non-functional genes, resulting in the loss of one of these genes and the *URA3* gene (i.e., "pop-out").
20 Thereafter, it is possible to positively select for strains lacking a functional *URA3* gene by plating cells on medium containing a non-toxic analog of a uracil pathway intermediate, 5-fluoro-orotic acid (5-FOA), which, when metabolized by *URA3⁺* strains, produces a
25 compound toxic to the cells. Because *URA3⁻* strains blocked at a specific point in the uracil pathway do not metabolize 5-FOA, they are not subjected to its toxic effects, and can thus be referred to as "5-FOA resistant". In contrast, *URA3⁺* strains metabolize 5-FOA
30 to produce a toxic compound which will prevent growth of the *URA3⁺* cells. The resulting *URA3⁻* cells that also lack the functional target gene are deficient in proteolytic activity. Because the *URA3⁻* phenotype is restored, the resulting cells can be transformed again using the *URA3*
35 gene as a selectable marker.

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The ability to positively select strains lacking a functional *URA3* gene employing a toxic analog of a uracil pathway intermediate allows the use of this very convenient "pop-out" method for imparting multiple phenotypic changes in *Pichia* hosts.

URA3⁻ *Pichia* strains which are also deficient in proteolytic activity, relative to the proteolytic activity present in wild-type strains of the same species, are particularly useful for transformation with expression vectors which contain an intact form of the *URA3* gene, and a gene encoding a proteolytically sensitive product (either as part of the same vector, or as a second vector which is transformed into the host). Those transformants which return to uracil prototrophy (which can be readily determined by simple screening procedures) should have incorporated therein the gene encoding a proteolytically sensitive product, and thus would be directly applicable to product expression.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

EXAMPLE I: ISOLATION OF THE *P. PASTORIS* PEP4 GENE

The *P. pastoris* PEP4 gene was identified in a bacteriophage lambda-based EMBL3 *P. pastoris* genomic DNA library by its ability to hybridize with a radiolabeled fragment of the homologous *Saccharomyces cerevisiae* PEP4 gene. The *P. pastoris* PEP4 gene was cloned by isolating positive plaques containing the hybridizing recombinant phage DNA.

A. Construction of a *P. pastoris* EMBL3 Genomic DNA Library

Bacteriophage λ was used as a vehicle for cloning the *P. pastoris* PEP4 gene. Fragments of a partial Sau3A digest of *P. pastoris* genomic DNA were inserted into the bacteriophage λ vector EMBL3 [Frischauf, A.-M. et al.

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(1983). J. Mol. Biol. 170:827], which contains elements of the bacteriophage λ genome essential for propagation of the recombinant DNA in bacterial hosts. The *P. pastoris* DNA-containing EMBL3 vectors were packaged in vitro into infectious virions to yield a bacteriophage λ *P. pastoris* genomic DNA library. Amplification of the library was achieved by propagation of the recombinant DNA in *Escherichia coli* host cells that had been infected with the recombinant virus.

10 *Pichia pastoris* genomic DNA (from strain NRRL Y-11430, from the Northern Regional Research Center, Peoria, IL) isolated using a glass rod swirl technique [Cregg et al. Mol. Cell. Biol. 5:3376-3385 (1985)] was digested with Sau3A at an effective concentration of 0.1 u/ μ g in 7, 14, 21 and 28 minute incubations conducted at 15 37°C. An aliquot from each incubation mixture was electrophoretically separated on a 1% agarose gel to determine the sizes of the digested DNA fragments. Digests incubated for 7 and 14 minutes appeared to 20 consist primarily of 9-23 kb fragments. These digests were pooled and ligated to EMBL3 vector arms, prepared as described below.

EMBL3 vector arms were prepared by double digestion of the vector (obtained from EMBL3 Cloning Kit, 25 Stratagene Cloning Systems, San Diego, CA; catalog #241211) with BamHI and EcoRI. The small BamHI/EcoRI linker that separates the arms from the stuffer fragment was removed from the digest by selective precipitation with ethanol. Ligation of the Sau3A-digested *Pichia* 30 genomic DNA (0.5 μ g) to 1 μ g of EMBL3 pre-digested arms was accomplished by incubation of the 5- μ l reaction mixture at 4°C for two days.

The recombinant bacteriophage λ DNA prepared by ligation of *P. pastoris* genomic DNA fragments and EMBL3 35 vector arms was packaged in vitro using commercial

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packaging extracts (Stratagene EMBL3 Cloning Kit). The EMBL3-based *P. pastoris* genomic library was amplified by plating the recombinant phage along with the *E. coli* lysogenic host strain P2 392 (provided in Stratagene EMBL3 Cloning Kit) which contains prophage P2. Wild-type bacteriophage do not grow in *E. coli* strain P2 392. Recombinant EMBL3-based bacteriophage, which lack two of the wild-type genes that confer P2 sensitivity, are able to grow well in this P2-containing *E. coli* strain. The use of *E. coli* P2 392 as the host strain in the amplification ensured that only recombinant phage would be reproduced in the bacterial host.

All of the plates encompassing the EMBL3-based *P. pastoris* genomic DNA library were overlayed with SM buffer (5.8 g NaCl, 2g MgSO₄·H₂O, 50 ml 1M Tris·HCl, pH 7.5, and 5 ml 2% gelatin per liter). After five hours, the supernatants were collected and pooled, and the titer and genome equivalents were calculated according to the manufacturer's instructions. The library contained approximately 10 genome equivalents, and its titer was 6 X 10¹¹ plaque-forming units/ml (pfu/ml).

B. Screening of the EMBL3 *P. pastoris* Genomic DNA Library Using the *S. cerevisiae* PEP4 Gene as a Probe

In order to adequately screen the *Pichia* genome for the PEP4 gene, 50,000 recombinant phage and the *E. coli* lysogenic host strain LE 392 (provided in Stratagene EMBL3 Cloning Kit) were plated onto four large 150-mm plates. After 6-7 hours of growth, the plates were chilled to 4°C. Each plate was marked and duplicate plaque lifts of each plate were prepared by placing nitrocellulose onto each plate. The filters were denatured, neutralized, baked and probed with the *S. cerevisiae* PEP4 gene [a gel-purified, ³²P-labeled 4.0 kb fragment of *S. cerevisiae* DNA containing the *S. cerevisiae* PEP4 gene obtained from the laboratory of Thomas Stevens, University of Oregon, Eugene, Oregon; see

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Rothman et al., Proc. Natl. Acad. Sci. USA 83: 3248-3252 (1986)]. Hybridization was conducted at 37°C in a solution containing 30% formamide, 6 X SSC, 5 X Denhardt's solution, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 µg/ml salmon sperm DNA. After hybridization, the filters were washed three times at room temperature using 2 X SSC and 0.1% SDS. Following these initial washes, the filters were then washed twice at 55°C using 2 X SSC and 0.1% SDS.

10 Fifteen positive plaques containing DNA that hybridized to the fragment of the *S. cerevisiae* PEP4 gene were identified in duplicate from autoradiograms of the filters. The area around each of the 15 positive plaques was isolated and placed in SM buffer. Six of the
15 isolates were plated at dilutions of 10^{-5} and 10^{-7} with *E. coli* strain LE 392 onto smaller 100-mm plates. Single plaque lifts of each plate were probed with the *S. cerevisiae* PEP4 gene fragment under the same hybridization and wash conditions used in the first
20 plaque screening. In this second round of screening, 12 positive plaques were detected on the autoradiogram. Nine of these single plaques were isolated and placed in SM buffer. Each of these nine plaques was plated at dilutions of 10^{-5} and 10^{-7} with *E. coli* strain LE 392 onto
25 small 100-mm plates. Again, single plaque lifts of each plate were probed with the *S. cerevisiae* PEP4 gene fragment under the same hybridization and wash conditions used in the first two screenings. Each plate contained approximately 10-20 plaques distributed evenly across the
30 plate. Autoradiograms of the filters revealed that every plaque on each plate hybridized to the PEP4 probe.

Five separate plaques from different plates were isolated and placed in SM buffer. DNA from large-scale cultures of three of these isolates, designated 4721,
35 5111 and 5131, respectively, was prepared using the

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induction method of bacteriophage isolation [Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1982)] in order to
5 identify, characterize and subclone the *PEP4* gene contained in the recombinant phage.

C. Characterization of the Insert in Isolates of the EMBL3 *P. pastoris* Genomic DNA Library that Hybridized to the *S. cerevisiae* *PEP4* Gene

10 Recombinant phage DNA prepared from the three isolates, referred to above, from the EMBL3 *Pichia* genomic DNA library (4721, 5111 and 5131), were digested with various restriction endonucleases, separated on a 0.8% agarose gel and visualized by ethidium bromide
15 staining. In addition, 1 μ l aliquots of these digests were separated on a second agarose gel which was blotted onto nitrocellulose and probed with the radiolabeled *S. cerevisiae* *PEP4* gene fragment. Hybridization was conducted at 37°C in a solution containing 30% formamide,
20 6 X SSC, 5 X Denhardt's solution, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 μ g/ml salmon sperm DNA. The filter was then washed in three 5-minute washes at room temperature with 2 X SSC and 0.1% SDS followed by two 5-minute washes at 55°C with 2 X SSC and 0.1% SDS.

25 Identical digests of DNA from two of the clones, 5111 and 5131, yielded the same pattern of restriction enzyme fragments, as determined by ethidium bromide staining, whereas the same digest of DNA from the third clone, 4721, yielded a different fragment pattern.
30 Analysis of the restriction enzyme fragments of DNA from each clone by Southern blot hybridization to the *S. cerevisiae* *PEP4* gene fragment revealed that the two classes of clones both contained a series of hybridizing fragments of the same size indicating that the two
35 classes of clones had a common overlapping DNA sequence that hybridized with the probe.

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D. Subcloning and Characterization of the Cloned *P. pastoris* PEP4 Gene

As determined by Southern blot hybridization of EcoRI-digested *P. pastoris* genomic DNA using the homologous *S. cerevisiae* PEP4 gene as a probe, the *P. pastoris* PEP4 gene is contained within a 10.6 kb EcoRI fragment of the *P. pastoris* genome. Southern blot hybridization of EcoRI-digested DNA of clone 4721, as described in Example 1C, revealed that it contained a 10.6 kb fragment that hybridized to the *S. cerevisiae* PEP4 gene. To facilitate manipulation of the cloned *P. pastoris* PEP4 gene, *P. pastoris* genomic DNA contained on an EcoRI fragment of DNA from isolate 4721 was subcloned into pUC19. Clone 4721 (25 µg) was digested with EcoRI (60 units) in a total volume of 300 µl. The digested DNA was separated on a 0.65% agarose gel, and the 10.6 kb EcoRI fragment was isolated with DE81 paper. The purified fragment was washed from the paper with 400 µl of 1 M NaCl and extracted with phenol/chloroform. The DNA was then precipitated with ethanol and resuspended in water to a total volume of 10 µl. Approximately 50 ng of the 10.6 kb fragment were ligated with an equal amount of pUC19 which had been cut with EcoRI and dephosphorylated. The ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of the diagnostic 10.6 kb EcoRI fragment. A large-scale plasmid preparation was made from a colony containing the correct plasmid, which was named pEP202. Plasmid pEP202 contains the complete *P. pastoris* PEP4 gene (see Figure 2).

To facilitate sequence analysis of the cloned *P. pastoris* PEP4 gene, a portion of the *P. pastoris* PEP4 gene was subcloned into pUC19. Plasmid pEP202 was digested with BamHI and EcoRI. The reaction mixture was separated on a 0.7% agarose gel, and the 0.45 kb BamHI

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fragment of DNA (see Fig. 2) was isolated using DE81 paper. The purified fragment was ligated to pUC19 (~20 ng) that had been linearized by digestion with BamHI and dephosphorylated. The ligation mixture was used to
5 transform *E. coli* strain MC1061. Transformants were selected for ampicillin resistance and screened by analysis of restriction enzyme digests of colony DNA for the presence of a single BamHI fragment. A single colony arising from this transformation was found to contain the
10 appropriate DNA construct, and was named pEP205 (see Figure 3).

Sequence analysis of plasmid pEP202 identified a DNA sequence with ~70% homology to the *PEP4* gene of *S. cerevisiae*. The amino acid sequence encoded by this DNA
15 sequence of pEP202 is 69% homologous to that encoded by the *S. cerevisiae PEP4* gene.

EXAMPLE II: DEVELOPMENT OF A *PEP4*-DEFICIENT (*PEP4*) STRAIN OF *P. PASTORIS*

20 **A. Construction of the *P. pastoris PEP4* Gene Disruption Vector pDR401**

Vector pDR401 was constructed for use in developing a *PEP4*-deficient (*PEP4*⁻) strain of *P. pastoris*. This vector contains a defective *P. pastoris PEP4* gene, which,
25 when used to transform *PEP4* strains of *P. pastoris*, integrates into the host genome by replacement of the wild-type *PEP4* gene.

pDR401 was constructed in a two-step procedure as follows. In the first step, the base vector in the
30 construction of pDR401, base vector pEP301, was constructed from pEP202. Vector pEP301 contains pUC19 sequences and the cloned *P. pastoris PEP4* gene from pEP202. Plasmid pEP202 (15 µg) was digested with SacI. A 5.5 kb SacI fragment (the fragment extending from the
35 SacI linker clockwise to the SacI site at ~5:00, and containing all of the pUC19 sequence and the entire *PEP4* gene; see Figure 2) was isolated from a 0.7% agarose gel

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using DE81 paper. The fragment was eluted from the paper with 400 μ l of 1 M NaCl, extracted with 400 μ l of phenol/chloroform and precipitated with ethanol. This DNA was then ligated with itself in a volume of 100 μ l containing 1 μ l of ligase and 1 μ l (~10 ng) of DNA. The ligation mixture was incubated at room temperature for 1 hr and then used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of a single 5.5 kb BglII fragment. Plasmid DNA was prepared from a transformed colony of MC1061 that contained the correct plasmid, which was named pEP301 (Figure 4).

In the second step of the construction of pDR401, the *P. pastoris* *HIS4* gene was inserted into the *PEP4*-containing plasmid pEP301 to yield the final vector. The *P. pastoris* *HIS4* gene was isolated on a 2.6 kb BglII fragment derived from pYJ8 Δ Cla [Cregg, J. et al. Mol. Cell. Biol. 5:3376-3385 (1985)]. Plasmid pYJ8 Δ Cla (15 μ g) was digested with BglII and the digested DNA was separated on a 0.7% agarose gel. The *HIS4* gene-containing 2.6 kb fragment was isolated with DE81 paper, eluted with 400 μ l of 1 M NaCl, extracted with 400 μ l of phenol/chloroform, precipitated with ethanol and resuspended in 10 μ l of water. Prior to inserting this 2.6 kb BglII fragment into the unique BglII site of pEP301, approximately 20 μ g of pEP301 were digested with BglII, dephosphorylated and extracted with phenol/chloroform. The 2.6 kb *HIS4*-containing fragment was then inserted into pEP301 by ligation of approximately 50 ng of the fragment to approximately 50 ng of the BglII-digested pEP301 in a total volume of 10 μ l containing 1 μ l of buffer, 1 μ l of ligase and water. Ligation was conducted at room temperature for 3 hrs and the ligation mix was used to transform MC1061 cells. Plasmid DNA

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prepared from an ampicillin-resistant colony was digested with BglII, SalI, BglII/SalI, PvuI, NcoI and KpnI to confirm the construction of pDR401. The restriction fragment pattern was consistent with that expected for the correct plasmid pDR401 (see Figure 5). Plasmid pDR401 is pUC19 with the *P. pastoris* *HIS4* gene inserted at the unique BglII site within the *PEP4* structural gene, thus disrupting it.

10 B. Transformation of *HIS4* *P. pastoris* Strain GS115 with a Fragment of pDR401

In order to create a *PEP4* strain of *P. pastoris*, the *HIS4* *PEP4* *P. pastoris* strain GS115 (ATCC 20864) was transformed with 20 µg of the 5.3 kb EcoRI/SacI fragment of pDR401 according to the spheroplast method (see US patent 4,879,231). This fragment of pDR401 consists of the *HIS4* gene-containing defective *PEP4* gene. Transformant strains resulting from this type of integration are prototrophic and can be distinguished from untransformed cells on this basis. The frequency of transformation was approximately $10^3 \mu\text{g}^{-1}$ DNA.

20 C. Characterization of Transformants

1. Analysis of transformant carboxypeptidase Y activities

His⁺ transformants were subsequently analyzed for carboxypeptidase Y activity using a colony overlay colorimetric screening procedure [see Jones, E. in Genetics 85: 23-33 (1977)]. In this assay, the His⁺ transformant cells were released from the transformation agar plates and grown on YEPD (yeast extract, 1% peptone, 2% dextrose and 2% agar) plates at a density of ~300 colonies per plate. The plates were overlaid with 0.6% agarose containing 40% dimethylformamide (DMF) to permeabilize the cells, and 1.2 mg/ml of the substrate APNE (N-acetyl DL phenylalanine β-naphthyl ester). Because the cells were permeabilized, some of the vacuolar content of the cell was accessible to the

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reagent APNE. After the agarose overlay had solidified, the plates were soaked in a solution of 5 mg/ml Fast garnet salt. APNE is cleaved by the esterolytic activity of carboxypeptidase Y. The products of this reaction
5 bind the fast garnet salt to produce a red color in the colony. Colonies lacking carboxypeptidase Y activity do not bind the salt and therefore stain less intensely than do colonies that possess this activity. *PEP4*⁺ colonies developed a red/pink center during the first 10-15
10 minutes after exposure to the garnet salt. In contrast, colonies defective at the *PEP4* locus were slow to develop this color and were distinguished as pink relative to the red *PEP4*⁺ colonies. Colonies that appeared to have low carboxypeptidase Y activities based on the results of
15 this assay (i.e., colonies that failed to develop a strong red color indicative of *PEP4*⁺ colonies) were isolated, transferred to a master plate, subcultured along with control colonies and re-screened using the overlay assay. Twenty colonies which again failed to
20 develop a strong red color were selected for analysis by Southern blot hybridization to determine if the *PEP4* locus of these transformants had been disrupted by integration of the fragment of vector pDR401.

2. Southern blot hybridization analysis

25 Genomic DNA was extracted from 20 transformant strains that exhibited low carboxypeptidase activity, designated p1-p20, and digested with SacI and EcoRI. This procedure should liberate the *HIS4*-containing defective *PEP4* gene as the 5.3 kb EcoRI/SacI fragment that was used
30 to transform the strains. Two Southern blot filters were prepared from these digested DNAs; one blot was probed with a radiolabeled 1.4 kb XbaI/EcoRV fragment from pEP301 (see Fig. 4), which contained a portion of the cloned *P. pastoris PEP4* gene and the other blot was
35 probed with a radiolabeled 2.6 kb BglII fragment of

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pDR401 containing the *HIS4* gene. Control DNA from the transformation host strain GS115, which had been digested with SacI and EcoRI, was included in this analysis for comparative purposes.

5 Digestion of genomic DNA from GS115 with SacI and EcoRI yielded a 2.9 kb fragment that hybridized to the portion of the *PEP4* gene contained in the radiolabeled XbaI/EcoRV fragment of pEP301. In contrast, this probe hybridized to fragments of a different size in
10 SacI/EcoRI-digested DNA from 19 of the 20 transformants analyzed. Only DNA from strain p17 yielded a hybridization pattern identical to that of DNA from the parental strain. The remaining 19 strains lacked a 2.9 kb hybridizing fragment characteristic of an undisrupted
15 *PEP4* locus and contained an approximately 5.3 kb fragment and/or larger fragments that hybridized to the *PEP4* gene probe. The 5.3 kb fragment was the same size as the transforming DNA released from vector pDR401 upon digestion with SacI and EcoRI.

20 The results of Southern blot hybridization of DNA from strains p1-p16 and p18-p20 revealed that these strains contained a defective *PEP4* gene with an intact *HIS4* gene therein, and that the *PEP4* locus of the strains had been disrupted. Strain p13 was grown in a one-liter
25 fermentation, as described in Example III, in order to analyze the proteolytic activity of the broth of a larger culture of a *PEP4* strain of *P. pastoris*.

3. Analysis of the transformant proteinase A activities

30 a. Protocol

The proteinase A activities of eight transformant strains were evaluated using an enzyme assay based on the method of Jones et al. [Genetics 102:655 (1982)]. Several control strains were also evaluated in this
35 assay: *PEP4* and *PEP4* strains of *S. cerevisiae* (strains DBY747 and 20B12, respectively, from the Yeast Genetic

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Stock Center, University of California, Berkeley, CA) and a *PEP4* wild-type strain of *P. pastoris* (strain NRRL Y-11430 from the Northern Regional Research Center, Peoria, IL).

5 Proteinase A is a vacuolar enzyme responsible for the aspartyl protease activity encoded by the *PEP4* gene in *S. cerevisiae*. The procedure used to evaluate the proteinase activities of transformant cell extracts is based on the measurement of proteinase A-mediated release
10 of amino acids from acid-denatured hemoglobin. Transformant cell extracts were incubated with acid-denatured hemoglobin, and the proteinase A activity present in the extract was determined by estimating the difference in the amount of amino acid released at time
15 zero and after 90 minutes of incubation.

Cultures of the *S. cerevisiae* control strains DBY747 (*PEP4*) and 20B12 (*PEP4*), the *PEP4 P. pastoris* strain NRRL Y-11430 and the experimental *PEP4* strains of *P. pastoris* were grown to stationary phase in YEPD medium. Cultured
20 cells (20 OD₆₀₀ units) were washed in 10 mM sodium azide and then lysed in 400 μ l of 100 mM Tris, pH 7.5, by vortexing the cells with acid-washed glass beads for one minute. The lysed cells were centrifuged in Eppendorf tubes for 10 minutes to remove cell debris. The
25 supernatant obtained after centrifugation (crude extract) was then examined for proteinase A activity as follows. Acid-denatured 1% hemoglobin (400 μ l) was added to 50 μ l of crude extract and incubated for 90 minutes at 37°C. Reactions were stopped by the addition of 0.2 ml of 1 N
30 perchloric acid. Insoluble material was removed by centrifugation, and 200 μ l of 0.31 M NaCl was added to 200 μ l of supernatant. A 40 μ l aliquot of this solution was then assayed using the Pierce BCA protein assay kit (see, for example, US Patent No. 4,839,295) for free
35 amino acids. The amount of free amino acids present in

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the sample that had been incubated for 90 minutes was compared to the amount present in a blank which consisted of a sample of a reaction mixture that was stopped at zero minutes. The relative difference in free amino acids between these two samples is a measure of proteinase A activity.

b. Results

The results of proteinase A assays of control and transformant strains (see Table I; ΔOD is a measure of the concentration of free amino acids in the sample) indicate that the proteinase A activity of the *PEP4* strain of *S. cerevisiae* represents only 10% of that of the *PEP4* strain of *S. cerevisiae*. Similarly, the proteinase A activities of the *PEP4* transformant strains (strains p1, p2, p5, p8, p13, p16 and p20) also are only approximately one-tenth of that of the *PEP4* strain of *S. cerevisiae*. The *PEP4* wild-type strain of *P. pastoris* displayed approximately half of the proteinase A activity of the *PEP4* strain of *S. cerevisiae*.

TABLE I
PROTEINASE A ASSAY RESULTS

Strain	Phenotype	$\Delta OD/\mu g$ protein
DBY747 (<i>S. cerevisiae</i>)	<i>PEP4</i> ⁺	28.1
20B12 (<i>S. cerevisiae</i>)	<i>PEP4</i> ⁻	2.7
<i>P. pastoris</i> control (NRRL Y-11430)	<i>PEP4</i> ⁺	13.1
p13	<i>PEP4</i> ⁻	3.3
p20	<i>PEP4</i> ⁻	4.2
p17	<i>PEP4</i> ⁺ (?)	7.5
p16	<i>PEP4</i> ⁻	0
p16	<i>PEP4</i> ⁻	0
p13	<i>PEP4</i> ⁻	3.3
p8	<i>PEP4</i> ⁻	3.3
p5	<i>PEP4</i> ⁻	5.0
p2	<i>PEP4</i> ⁻	6.6
p1	<i>PEP4</i> ⁻	6.0

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The data obtained in proteinase A assays of *PEP4* *P. pastoris* strains generated by transformation of a *PEP4* strain with a defective *PEP4* gene are consistent with the results of Southern blot analyses of DNA from these transformants which indicate that the *PEP4* locus of the

5 transformants was disrupted upon transformation.

EXAMPLE III: FERMENTATION OF A *PEP4* STRAIN OF *P. PASTORIS*

A. Procedure

A *PEP4* strain of *P. pastoris*, p13, generated by transformation of strain GS115 with a defective *PEP4* gene-containing *SacI*/*EcoRI* fragment of vector pDR401, was grown in a one-liter fermentation according to a three-phase protocol consisting of a glycerol batch growth phase, a limited glycerol fed-batch phase and a methanol

10 fed-batch phase as follows.

15

A two-liter fermentor was autoclaved with 1000 ml of minimal salts medium (21 ml 85% phosphoric acid, 0.9 g calcium sulfate·2H₂O, 14.3 g potassium sulfate, 11.7 g magnesium sulfate and 3.2 g potassium hydroxide) and 2% glycerol. After sterilization, 4 ml PTM₁ trace salts solution (6 g/l cupric sulfate·5H₂O, 0.8 g/l sodium iodide, 3 g/l manganese sulfate·H₂O, 0.2 g/l sodium molybdate·2H₂O, 0.02 g/l boric acid, 0.5 g/l cobalt chloride, 20 g/l zinc chloride, 65 g/l ferrous

20 sulfate·H₂O, 0.2 g/l biotin and 5 ml sulfuric acid) were added to the fermentor and the pH was adjusted to 5 with concentrated NH₄OH. The pH of the medium was maintained at 5 by addition of 50% NH₄OH containing 0.1% Struktol J673 antifoam. Inocula were prepared from buffered yeast

25 nitrogen base (YNB) glycerol plates (phosphate-buffered YNB, 2% glycerol, 2% agar) and grown overnight at 30°C in phosphate-buffered YNB (11.5 g/L KH₂PO₄, 2.66 g/L K₂HPO₄, 0.67% yeast nitrogen base, pH 5) containing 2% glycerol. The fermentor was inoculated with 10-50 ml of the

30

35 cultured cells which had grown to an OD₆₀₀ of 1-8, and the

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batch growth regimen was continued for approximately one day until glycerol was exhausted. At the point of glycerol exhaustion, as indicated by increased dissolved oxygen, a glycerol feed (50% glycerol plus 12 ml/L of PTM₁) was initiated at 10 ml/h and continued until 40 ml of glycerol feed had been added. After termination of the glycerol feed, a methanol feed (100% methanol plus 12 ml/L PTM₁) was started at an initial rate of approximately 2 ml/h. After 3 hours, the methanol feed rate was increased to 6 ml/h. The methanol feed rate was maintained at 6 ml/h for 12-18 hours and was then increased to 10 ml/h and maintained at 10 ml/h for the duration of the fermentation. The vessel was harvested after 400 ml of methanol had been added to the fermentor.

15 **B. Sample Preparation**

Samples (15 ml aliquots) of the fermentor culture were removed from the fermentor at various time intervals throughout the course of the fermentation. Aliquots of each sample were centrifuged at 6500 x g for 5 minutes to separate broth and cells. The levels of the NH₄OH, antifoam, glycerol, and methanol reservoirs were recorded at these time points. Methanol and ethanol concentrations in the supernatant were determined by gas chromatography using a PorapakQ column (Alltech).

25 In addition, the wet weight of the culture was determined as an indicator of cell growth in the fermentor. For this purpose, a one ml aliquot of the fermentor culture was centrifuged for four minutes in a microfuge, the supernatant was decanted, and the wet pellet was weighed.

30 **C. Results**

Growth of the PEP4 strain of *P. pastoris* p13 in a one-liter fermentation was monitored by determining the wet cell weight of the fermentor culture (in g/l) at various times during the fermentation. A time course of

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the growth of strain p13 during the methanol fed-batch phase of the fermentation, when compared with the time course of the growth of the *HIS4 PEP4* strain G+PA0804H2 (generated by transformation of the *HIS4 PEP4 P. pastoris* strain GS115 with an expression vector containing the wild-type *HIS4* gene) during a similar one liter fermentation, demonstrates that the growth capabilities of the *PEP4* strain of *P. pastoris* are comparable to those of a *PEP4* strain.

10 **EXAMPLE IV: ANALYSIS OF THE PROTEOLYTIC ACTIVITY OF THE BROTH OF A PEP4 STRAIN OF P. PASTORIS GROWN IN A ONE-LITER FERMENTATION**

To determine if disruption of the *P. pastoris PEP4* gene was associated with a change in the proteolytic activity of the broth of *P. pastoris*, the proteolytic activities of the broths from one-liter fermentations of a *PEP4* strain, strain p13, and a *PEP4* strain were compared. In this study, two different peptides, epidermal growth factor (EGF; a recombinantly synthesized molecule consisting of the first 52 amino acids of the authentic 53 amino acid EGF molecule, as described in US patent application Serial No. 323,964) and growth hormone releasing factor (GRF; recombinantly synthesized as described in EP 206783) were separately incubated at room temperature in cell-free broth from the one-liter fermentation of the *PEP4 P. pastoris* strain p13, and in the cell-free broth from a similar one-liter fermentation of the *HIS4 PEP4 P. pastoris* strain G+PA0804H2. After incubation for a specified period, aliquots of each incubation mixture were examined by reverse phase high performance liquid chromatography (HPLC), described below, to determine the amount of intact peptide remaining in each sample and thereby determine the extent of proteolytic degradation of the peptide.

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A. Reverse-Phase High-Performance Liquid Chromatography (HPLC)

The reverse-phase HPLC system used in the analysis of EGF and GRF peptides in buffer and broth from fermentations of *P. pastoris* strains included a Waters 600 (Bedford, MA) solvent delivery system, Waters Model 481 Lambda Max variable wavelength detector, Wisp 710B autoinjector and a Shimadzu Chrom-Pac integrator (Cole Scientific, Moorepark, CA). Samples of broth from the fermentations of the *PEP4 P. pastoris* strain p13 and the *HIS4 PEP4 P. pastoris* strain G+PA0804H2 were diluted 1:10 in 0.1 M sodium phosphate, pH 5.0. Fifteen microliters of concentrated GRF stock was added to 285 μ l of diluted broth and incubated for four hours. A similar dilution of GRF stock in the phosphate buffer was also incubated for four hours as a control. Sixty microliters of EGF stock were added to 240 μ l of diluted broth or buffer and incubated for eight hours. Samples of each incubation mixture were separately injected into a Waters μ Bondapak C18 reverse phase column. The peptides were eluted from the column in a 20-minute linear gradient of 20-60% mobile phase B (95% acetonitrile, 5% water, 0.1% trifluoroacetic acid). Mobile phase A (0.1% trifluoroacetic acid) was used to dilute mobile phase B in preparing the elution gradient.

B. Results

The amount of intact peptide (of the EGF or GRF molecules that were incubated in the fermentation broth of the *PEP4 P. pastoris* strain p13 and the broth of the *PEP4 P. pastoris* strain G+PA0804H2) was evaluated by comparing chromatograms obtained in HPLC analyses of intact EGF or GRF contained in 0.1 M sodium phosphate buffer, pH 5.0, and of EGF or GRF contained in broth. Chromatograms from HPLC analyses of the standard intact peptides consist of a major peak reflecting the amount of the standard peptide present in the sample and the

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retention time characteristic of the peptide. In contrast, proteolytic fragments of either peptide are retained on the HPLC column for varying lengths of time that differ from the retention time associated with the intact peptide. Therefore, chromatograms from HPLC analysis of proteolytic fragments of either peptide (EGF or GRF) differ from chromatograms generated in HPLC analyses of intact peptides in terms of the number and sizes of the peaks and the retention times associated with the fragmented species. Based on these differences, it was possible to estimate the amount of intact EGF or GRF peptide in the broth incubation samples.

Based on HPLC analyses of GRF and EGF samples incubated in *PEP4 P. pastoris* control broth, it has been determined that less than 10% of each of the two peptides remains intact after incubation in broth from the fermentation of the *PEP4* strain G+PA0804H2. In contrast, the level of proteolytic degradation of these peptides in the broth of the *PEP4 P. pastoris* strain is significantly less than that in the broth of the *PEP4* strain (GRF remained >60% intact, even after 4 hr incubation; EGF remained >90% intact, even after 8 hr incubation). These data demonstrate that disruption of the *PEP4* gene of *P. pastoris* results in a substantial reduction of the proteolytic activity in the broth of the strain.

EXAMPLE V: ISOLATION OF THE *P. PASTORIS* URA3 GENE

The *P. pastoris* URA3 gene was identified in a plasmid (YEpl3)-based *Pichia* genomic library by its ability to complement the pyrF mutation (corresponding to a defect in the orotidine monophosphate decarboxylase activity) in *E. coli* strain CSH-28. The *P. pastoris* URA3 gene was cloned by isolating colonies of *E. coli* strain CSH-28 that had been transformed with library DNA and were capable of growth on media lacking uracil.

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A. *P. pastoris* YEp13 Genomic DNA Library

Plasmid YEp13 [Broach et al., Gene 8: 121-133 (1979)] is a convenient shuttle vector that contains an origin of replication for both *S. cerevisiae* (2 μ replicon) and *E. coli* (pBR ori). In addition, YEp13 contains the Amp^R (ampicillin resistance) gene for use as a selectable marker for transformation of *E. coli* and the *LEU2* gene (a leucine biosynthetic pathway gene) for use as a selectable marker in *S. cerevisiae*. A *P. pastoris* (strain NRRL Y-11430) genomic DNA library has been prepared using plasmid YEp13, as described by Cregg et al. [*Mol. Cell. Biol.* 5: 3376-3385 (1985)].

B. Screening of the *P. pastoris* YEp13 Genomic DNA Library for the *URA3* Gene

The *pyrF* *E. coli* strain CSH-28 [see Miller, J. H., in *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972)] is defective for orotidine-5'-phosphate decarboxylase activity and requires uracil when grown on defined medium. It has been demonstrated that the *S. cerevisiae* *URA3* gene can complement the *pyrF* mutation in *E. coli* [Rose, M., Grisafi, P. and Botstein, D. *Gene* 29:113-124 (1984)]. Therefore, *E. coli* strain CSH-28 was transformed with DNA from the *P. pastoris* YEp13 genomic DNA library in order to screen the library for the *P. pastoris* *URA3* gene capable of complementing the *pyrF* mutation of the strain.

Transformed CSH-28 cells were plated onto a semi-defined medium which did not contain uracil. Untransformed cells would not grow on this medium. CSH-28 transformants (transformed with *P. pastoris* genomic library DNA) capable of growing on plates lacking uracil arose at a frequency of ~10/ μ g of transforming DNA. Plasmid DNA was isolated from ten of the transformants that did not require uracil for growth. These plasmids were used to transform *E. coli* strain

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CSH-28, and ten of ten plasmids complemented the uracil auxotrophy of this strain at high frequency. One of the selected transformants generated by transformation of CSH-28 with *P. pastoris* genomic library DNA harbored a
5 9.0 kb insert that contained a 6.6 kb SphI fragment. The 6.6 kb SphI fragment was subcloned into the SphI site of pUC19 for further analysis.

Plasmid DNA from this transformant was digested with SphI and subjected to electrophoresis on a 0.6% agarose
10 gel. The 6.6 kb fragment was isolated using DE81 paper and was eluted from the paper with 400 μ l of 1 M NaCl. DNA was extracted with 400 μ l of phenol/chloroform and precipitated with ethanol. The 6.6 kb fragment was then ligated with 10 ng of alkaline phosphatase-treated,
15 SphI-digested pUC19. The ligation mixture was used to transform *E. coli* MC1061 cells. Ampicillin-resistant transformants were screened by analysis of restriction enzyme-digested colony DNA for the presence of a 6.6 kb SphI fragment. The correct plasmid was called pPU201.
20 Plasmid pPU201 was used to transform CSH-28 and was able to complement the uracil auxotrophy of this strain.

C. Characterization of the Insert in Plasmid pPU201

A map of the restriction enzyme recognition sites of the 6.6 kb insert of *P. pastoris* DNA in plasmid pPU201
25 (Figure 6) was prepared by digesting pPU201 with a variety of enzymes and analyzing the resulting fragments using a DNA length computer program (MapSort; University of Wisconsin Genetics, Madison, WI) to determine the approximate sizes of the fragments. In order to
30 delineate the *URA3* gene contained in the 6.6 kb insert of pPU201, a 5 ng aliquot of each restriction enzyme digest of pPU201 was separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a radiolabeled 1.3 kb *Bgl*II fragment of the
35 *C. tropicalis* *URA3A* gene (see PCT Publication No. WO

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90/09449). The filters were hybridized to the probe at 27°C using a solution containing 25% formamide, 6x SSC, 5x Denhardt's solution, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and 100 µg/ml salmon sperm DNA. After hybridization, the filters were washed three times at room temperature using 1x SSC and 1% SDS for 5-10 minutes per wash, and then washed twice with 0.5x SSC and 0.5% SDS at 45°C for 10 minutes per wash. These low stringency conditions permitted hybridization between divergent *URA3* gene sequences. Additional samples of each digest of pPU201 were separated on an identical 1% agarose gel and stained with ethidium bromide for comparison of hybridizing and non-hybridizing fragments. Comparison of the hybridizing fragments and the restriction map of pPU201 made it possible to localize the *URA3* gene in pPU201 to the approximately 1.3 kb NcoI-SalI fragment as shown in Figure 6. With this knowledge, it was then possible to construct subclones suitable for sequencing and further characterization of the *P. pastoris URA3* gene.

Plasmid pPU202 (Figure 7) was constructed by digesting pPU201 with EcoRV and PstI, isolating the approximately 4.0 kb fragment containing the *URA3* gene, and ligating it into pUC19 at the SmaI and PstI sites. Plasmids pPU203, pPU205 and pPU206 (Figures 8-10) were constructed by digesting pPU202 with SacI, KpnI and EcoRI, respectively, and then religating in a large volume (200 µl). Because there is a recognition site for each of these enzymes in the cloned *P. pastoris* genomic insert DNA fragment as well as the pUC19 polylinker of pPU202, this strategy allowed for the convenient removal of DNA between these sites in pPU202. The resulting plasmids were then used to transform *E. coli* strain CSH-28 to determine whether or not each deletion construct could complement the pyrF mutation. The results

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indicated that pPU203 and pPU205, but not pPU206, contained a functional *URA3* gene allowing growth of the *pyrF* strain on defined medium lacking uracil. These findings are consistent with the mapped position of the *P. pastoris URA3* gene in pPU201.

The subclones of the *P. pastoris* genomic DNA fragment carrying the putative *URA3* gene were sequenced using the Sanger dideoxy method [see Sanger et al., Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977)]. The sequence for the structural gene and approximately 100 bp of flanking sequence was determined in both directions and is presented in Sequence ID No. 3. The amino acid sequence deduced from the cloned *P. pastoris URA3* gene (see Sequence ID No. 4) has 73% homology with the amino acid sequence deduced from the *S. cerevisiae URA3* gene, 71% homology with the amino acid sequence deduced from the *URA3A* and *URA3B* genes of *C. tropicalis* and 72% homology with the amino acid sequence deduced from the *URA3* gene of *Kleuveromyces lactis*.

EXAMPLE VI: DEVELOPMENT OF IGF-1-EXPRESSING PEP4-DEFICIENT (PEP4⁻) STRAINS OF PICHIA

A. Generation of IGF-1-Expressing PEP4⁻ Strains by Gene Addition

1. Construction of the *P. pastoris* PEP4 gene disruption vector pDR421

Plasmid pDR421 was constructed for use in the development of *PEP4*-deficient (*PEP4⁻*) strains of *Pichia pastoris* by disruption of a host *PEP4* gene through addition of an incomplete *PEP4* gene to the endogenous *PEP4* locus. This vector contains an internal portion of the *PEP4* gene, which, when used to transform *PEP4* strains of *P. pastoris*, integrates into the host genome at the *PEP4* locus to generate two incomplete and nonfunctional copies of the *PEP4* gene.

In order to generate the disruption vector pDR421, the *URA3* gene of *Pichia* was cloned into vector pEP205

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(consisting of pUC19 sequences and the portion of the *PEP4* gene contained in the ~450 bp *Bam*HI fragment derived from pEP202). This was achieved by subcloning the *URA3* gene from pPU205 (see Figure 9) as a 2 kb *Spe*I-*Sph*I DNA
5 fragment into the *Xba*I-*Sph*I sites of pEP205 (see Fig. 3).

Plasmid pPU205 was digested with *Spe*I and *Sph*I and the reaction mixture was separated on a 0.8% agarose gel. The 2 kb DNA fragment containing the *URA3* gene was
10 isolated from the gel using DE81 paper, eluted and purified. Plasmid pEP205 was digested with *Xba*I and *Sph*I. The 2 kb *URA3* gene-containing *Spe*I-*Sph*I fragment isolated from pPU205 was ligated to *Xba*I/*Sph*I-digested pEP205 and the mixture was used to transform *E. coli* strain MC1061 to ampicillin resistance. Ampicillin-
15 resistant colonies were screened by analysis of *Bam*HI/*Sph*I restriction enzyme-digested colony DNA for the presence of 2.7 kb, 0.4 kb and 1.9 kb diagnostic fragments. A transformant was found to harbor a plasmid with the correct DNA construct called pDR421 (Figure 11).

20 **2. Transformation of an IGF-1-expressing *URA3* *P. pastoris* strain (IGF-U) with pDR421**

The *URA3* IGF-1-expressing strain of *P. pastoris*, IGF-U, was transformed with pDR421 to generate *PEP4*, IGF-1-expressing strains of *P. pastoris*.

25 **a. Generation of IGF-U**

5-Fluoro-orotic acid (5-FOA) is an analog of a uracil biosynthetic pathway intermediate that, when metabolized by *Ura*⁺ strains, yields a toxic compound. Because the uracil biosynthetic pathway of *Ura*⁻ strains is
30 blocked at certain steps, these strains do not metabolize 5-FOA (to produce a compound toxic to the cells) and are therefore 5-FOA resistant. In contrast, *Ura*⁺ strains metabolize 5-FOA and cannot survive on 5-FOA-containing medium. Therefore, plating cells on 5-FOA-containing
35 medium can be used as a method to generate *Ura*⁻ strains by

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spontaneous mutation [see, for example, Boeke et al., Mol. Gen. Genet. 197: 345-346 (1984)].

A *URA3*⁻ derivative of the IGF-1-producing strain G+IMB206S1 [for a description of this strain, see
5 commonly assigned U.S. Patent Application Serial No. 07/578,728, filed September 4, 1990, which is hereby incorporated by reference herein in its entirety] was generated by direct plating of $\sim 5 \times 10^7$ cells this strain into 5-FOA-containing medium supplemented with uracil
10 (0.67% yeast nitrogen base, 2% agar, 2% glucose, 750 mg/l of 5-FOA and 48 mg/l of uracil). After one week of incubation at 30°C, a colony, designated IGF-U, growing on the plate was isolated. This colony, which required uracil in order to grow, was unable to complement a *URA3*
15 strain of *Pichia pastoris*.

b. Transformation of IGF-U

Approximately 20 µg of pDR421 was digested with *Bgl*II was used to transform IGF-U using the standard spheroplast transformation procedure. Transformants were
20 selected by their ability to grow in the absence of uracil over a 6 day period.

3. Characterization of transformants

a. Analysis of transformant carboxypeptidase Y activities

25 *Ura*⁺ transformants were subsequently analyzed for carboxypeptidase Y activity using a colony overlay colorimetric screening procedure, as described in Example II. Colonies of *Ura*⁺ transformants that appeared to have low carboxypeptidase Y activities based on the results of
30 this assay (i.e., colonies that failed to develop a strong red color indicative of *PEP4*⁺ colonies) were isolated, transferred to a master plate, subcultured along with control colonies and rescreened using the overlay assay. One colony which again failed to develop
35 a strong red color was called M+IMB206S1.

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b. Analysis of intact IGF-1 expression levels of an IGF-1-expressing PEP4 strain of P. pastoris grown in one- and ten-liter fermentations

5 i. Fermentation of an IGF-1-expressing PEP4 strain of P. pastoris

An IGF-1-expressing PEP4 strain of *P. pastoris*, M+IMB206S1, generated as described in Example VI.A.2.b., was grown in one- and ten-liter fermentations according to a three-phase protocol consisting of a glycerol batch
10 growth phase, a limited glycerol fed-batch phase and a methanol fed-batch phase. In order to compare the intact IGF-1 expression levels of PEP4 and PEP4 IGF-1-expressing strains of *P. pastoris*, two PEP4 strains of *P. pastoris*, G+IMB204S14 and G+IMB206S1, containing four and six
15 copies of an IGF-1 gene expression cassette, respectively (see, U.S. application Serial No. 07/578,728), were also grown in comparable fermentations as follows.

One-liter fermentation protocol

A two-liter fermentor (Biolafitte, Princeton, NJ)
20 was autoclaved with 900 ml of minimal salts medium (21 ml 85% phosphoric acid, 0.9 g calcium sulfate \cdot 2H₂O, 14.3 g potassium sulfate, 11.7 g magnesium sulfate, and 3.2 g potassium hydroxide) and 30 g of glycerol. After
sterilization, 4 ml PTM₁ trace salts solution (6 g/l
25 cupric sulfate \cdot 5H₂O, 0.08 g/l sodium iodide, 3 g/l manganese sulfate \cdot H₂O, 0.2 g/l sodium molybdate \cdot 2H₂O, 0.02 g/l boric acid, 0.5 g/l cobalt chloride, 20 g/l zinc chloride, 65 g/l ferrous sulfate \cdot H₂O, 0.2 g/l biotin and
5 ml sulfuric acid) were added to the fermentor and the
30 pH was adjusted to 5 with concentrated NH₄OH. The pH was controlled by addition of 50% NH₄OH containing 0.1% Struktol J673 antifoam (added to control foaming). The temperature was maintained at 30°C, and dissolved oxygen was maintained above 20% of saturation by increasing
35 agitation, aeration, or the supplementation of the air feed with oxygen.

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Inocula were prepared from cells grown overnight at 30°C in buffered YNB containing 2% glycerol. The fermentor was inoculated with 40-70 ml of the cultured cells which had grown to an OD_{600} of 2-8, and the batch growth regimen was continued for 18-24 hours until glycerol was exhausted. At the point of glycerol exhaustion, indicated by an increase in dissolved oxygen concentration, a glycerol feed (50% w/v glycerol plus 12 ml/L PTM_1) was initiated at 10 ml/hr. In pH 5.0 fermentations, the pH of the culture was maintained at 5 throughout the fermentation. In low pH fermentations (i.e., pH 2.8 or pH 3.5), the set point of the pH controller was adjusted to the desired pH after initiation of the glycerol feed. After four hours, the pH of the culture decreased to the set point value as a result of cellular metabolism. This lower pH was then maintained throughout the remainder of the fermentation. The glycerol feed was then terminated and a methanol feed (100% methanol plus 12 ml/L PTM_1) was initiated at a rate of 2 ml/hr. After three hours of methanol feeding, the feed rate was increased to 6 ml/hr and maintained at this rate for the remainder of the fermentation. The vessel was harvested 72 hours after initiation of the methanol feed.

The fermentation was monitored in terms of NH_4OH , antifoam, glycerol, methanol, ethanol, and wet cell weight levels as described in Example III. Broth and cell samples were collected throughout the fermentation as also described in Example III.

Ten-liter fermentation protocol

A 15-liter fermentor containing 3.5 liters of 10X basal salts (42 ml 85% phosphoric acid/l, 1.8 g calcium sulfate $\cdot 2H_2O$ /l, 28.6 g potassium sulfate/l, 23.4 g magnesium sulfate/l, 6.5 g potassium hydroxide/l) and 220 g glycerol in a total volume of 5.5 liters was

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sterilized. After the fermentor had cooled, 24 ml PTM₁ trace salts were added and the pH was adjusted to 5.0 with the addition of 28% ammonium hydroxide. The pH was controlled by the addition of the same solution. Foaming was controlled with the addition of a 5% solution of Struktol J673. Temperature was maintained at 30°C, and dissolved oxygen was maintained above 20% of saturation by increasing agitation, aeration, reactor pressure or by supplementation of the air feed with oxygen. Inocula were prepared from *P. pastoris* cells grown overnight in buffered yeast nitrogen base (YNB; 11.5 g/L KH₂PO₄, 2.66 g/L K₂HPO₄, 6.7 g/L yeast nitrogen base, pH 6) containing 2% glycerol. The fermentor was inoculated with 500-700 ml of the cultured cells which had grown to an OD₆₀₀ of 2-8, and the batch growth regime was continued for 18-24 hours. At the point of glycerol exhaustion, indicated by an increase in dissolved oxygen concentration, a glycerol feed (50% w/v glycerol plus 12 ml/L PTM₁) was initiated at 100 ml/hour and continued for 4 hours. The glycerol feed was then terminated and a methanol feed (100% methanol plus 12 ml/L PTM₁) was initiated at 20 ml/hr. With the initiation of the methanol feed, the set point of the pH controller was adjusted to 2.8. The pH then gradually decreased to the set point value as a result of cellular metabolism. After 4 hours of methanol feeding, the methanol feed rate was increased to 60 ml/hour and maintained at this rate for a total of approximately 72 hours, at which point the vessel was harvested.

30 ii. IGF-1 expression levels of PEP4 and PEP4 IGF-1-expressing strains

One of the several forms of IGF-1 produced in fermentations of recombinant IGF-1-secreting strains of *P. pastoris* is a nicked species consisting of two or more fragments of the IGF-1 molecule held together by

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disulfide bonds. The fragments are generated by proteolytic cleavage of one or more peptide bonds of the amino acid backbone of the IGF-1 molecule. Although nicked and intact IGF-1 molecules are indistinguishable on the basis of apparent molecular weight [under non-reducing conditions, as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)], these species can be resolved by reverse phase HPLC under non-reducing conditions and by SDS-PAGE under reducing conditions (i.e., in the presence of a reducing agent such as dithiothreitol). Reduction of the disulfide bonds holding the fragments of nicked IGF-1 together results in liberation of the individual proteolytically generated IGF-1 fragments which have smaller molecular weights than the intact molecule.

Quantitation of IGF-1 expression levels

The yields of nicked and authentic (intact, correctly folded, monomeric) IGF-1 in the cell-free broth were determined by quantitative reverse phase HPLC. The HPLC system that was used was the same as that described in Example IV, except a Vydac C4 column (0.46 x 5 cm) was employed instead of a C18 column. A 1%/minute gradient of 25-42% mobile phase B was passed through the column during a period of 17 minutes at a flow rate of 1 ml/minute to elute samples from the column. The detector was set at 0.05 absorbance units full scale (AUFS), and a wavelength of 215 nm was used for maximum sensitivity.

To distinguish the authentic and nicked IGF-1 species in *P. pastoris* broth by HPLC, it was necessary to clean-up the broth by removing some endogenous *P. pastoris* contaminants from the broth prior to loading broth samples onto the HPLC column. This was accomplished by passing the broth through a sulphopropyl-based cation exchange resin contained in a 0.25 ml column. The resin was first washed with 2 ml of 0.2 M

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acetic acid, then equilibrated with 2 ml of 0.02 M acetic acid. A volume of crude cell-free broth (1 ml) was loaded onto the column which was then washed with 1 ml of 0.02 M acetic acid. The IGF-1 was eluted with 2 ml of 0.02 M sodium acetate, pH 5.5, plus 1 M NaCl. The first 1 ml of eluate contained 75-80% of the total IGF-1 and was usually the only elution volume collected. The column was then regenerated by washing with 2 ml of 100% methanol and thereby available for re-use.

10 In order to quantitate the levels of *Pichia*-produced IGF-1, known amounts of standard IGF-1 (Amgen, Thousand Oaks, CA) were injected into the HPLC column and the area under the corresponding peaks in the chromatograms was measured. A standard curve was generated by plotting
15 area versus μg of IGF-1 loaded onto the HPLC column. A correlation coefficient for use in converting the area under HPLC chromatogram peaks to IGF-1 concentration was calculated from the standard curve. When the detector was set at 0.05 AUFS and a wavelength of 215 nm, the
20 correlation coefficient was 350 units/ μg of IGF-1 injected onto the column. Using this information, it was possible to determine the concentration of correctly folded, intact monomeric IGF-1 present in a cleaned-up broth sample by measuring the area under the
25 corresponding peak on the chromatogram from HPLC analysis of the sample. This correlation coefficient was also used to estimate the approximate concentration of the nicked IGF-1 species as well. However, the absolute concentrations of the nicked species may vary depending
30 on differences in the specific correlation coefficients of intact and nicked IGF-1.

Results of one-liter fermentations

One-liter low pH (pH 2.8) fermentations of the *PEP4* IGF-1-expressing strain consistently yielded greater
35 amounts of total monomeric (authentic plus nicked) IGF-1

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(~200-250 mg/l) than one-liter low pH fermentations of the *PEP4* IGF-1-expressing strains (~160-190 mg/L). Furthermore, the percentage of authentic IGF-1 in the broth of the *PEP4* strain was somewhat higher (77%) than that in the broth of the *PEP4* strains (65%). However, a much more dramatic difference in the monomeric IGF-1 production levels of the *PEP4* and *PEP4* strains was detected in pH 5.0 fermentations of these strains. Essentially no IGF-1 was detected in one-liter pH 5.0 fermentations of the *PEP4* IGF-1-expressing strains G+IMB204S14 and G+IMB206S1. This result indicates that the authentic IGF-1 produced in fermentations of *PEP4* strains is subjected to extensive proteolysis at pH 5.0, but to only limited proteolysis at lower pH. In contrast, one-liter pH 5.0 fermentations of the *PEP4* IGF-1-expressing strain M+IMB206S1 yielded at least 200 mg of monomeric IGF-1/l, approximately 80% of which was authentic IGF-1. The *PEP4* IGF-1-expressing strain thus appears to be significantly improved relative to the *PEP4* IGF-1-expressing strains for production of authentic IGF-1 at pH 5.0 and somewhat improved for production of authentic IGF-1 at pH 2.8.

Results of ten-liter fermentations

Ten-liter fermentations of the *PEP4* IGF-1-expressing strain of *P. pastoris* yielded greater amounts of total monomeric IGF-1 (~200 mg/l) than did ten-liter fermentations of the *PEP4* IGF-1-expressing strains (~170 mg/l).

The compositions of the total monomeric IGF-1 produced in 10-liter fermentations of the *PEP4* and *PEP4* strains also differed. Greater than 75% (164 mg/l) of the total monomeric IGF-1 in the 10-liter fermentation of the *PEP4* strain M+IMB206S1 was authentic IGF-1, whereas only about 50% (88 mg/l) of the total monomeric IGF-1 in

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the 10-liter fermentation of the *PEP4* strain G+IMB204S14 was authentic IGF-1.

Furthermore, because the cell yield in the fermentation of the *PEP4* strain was ~30% less than the cell yield in the fermentation of the *PEP4* strain, the per cell yield of authentic IGF-1 was greatly enhanced in the fermentation of the *PEP4* strain. As a consequence of lower cell yield in the fermentation of the *PEP4* strain, a greater volume of cell-free broth was recovered from the fermentation of the *PEP4* strain (relative to the volume of cell-free broth recovered from the fermentation of the *PEP4* strain). This results in the recovery of higher levels of secreted IGF-1 from the fermentation of the *PEP4* strains (relative to the amount of secreted IGF-1 recovered from the fermentation of the *PEP4* strain).

The results presented above demonstrate that the *PEP4* IGF-1-expressing strain is improved, relative to the *PEP4* IGF-1-expressing strain, for production of authentic IGF-1 on a large scale.

B. Generation of an IGF-1-Expressing *PEP4* Strain by Gene Replacement

1. Construction of the *P. pastoris* gene disruption vectors pDR601 and pDR602

Vectors pDR601 and pDR602 were used in the development of *PEP4*-deficient (*PEP4*⁻) strains of *P. pastoris* by disruption of a host *PEP4* gene through replacement of the endogenous *PEP4* gene with a defective *PEP4* gene. This vector was constructed in several steps as follows (see also diagram in Figure 13).

Plasmid pEP301 (see Figure 4), consisting of pUC19 sequences and the cloned *P. pastoris* *PEP4* gene from pEP202, was cleaved with *Nco*I, and the DNA was then precipitated with ethanol, harvested, resuspended and ligated in ligation reaction mixture. This digestion and ligation effectively removed an internal portion of the

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PEP4 gene contained in an ~0.5 kb NcoI fragment. After ligation, the DNA was digested with BglIII to linearize any remaining parental plasmid, and the DNA was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of a 0.5 kb NcoI fragment. The correct plasmid, containing the defective PEP4 gene lacking an ~0.5 kb NcoI fragment, was named pDL321. A second plasmid, pUC19XX, was generated by cleaving pUC19 with SmaI and HincII and religating, effectively removing a portion of the polylinker containing the BamHI and XbaI sites. Plasmid pUC19XX was then cut with SacI and EcoRI and ~10 ng was ligated with ~50 ng of the SacI/EcoRI 2.2 kb fragment of pDL321, which had been gel-purified and isolated with DE81 paper. The ligation mix was used to transform MC1061 cells, and ampicillin-resistant colonies were screened by analysis of BstEII/XbaI-digested colony DNA. Plasmid showing the correct digest pattern was designated pDL322.

pDL322 was then cut with XbaI and 10 ng were ligated with 10 ng of an oligonucleotide linker of the sequence 5'-CTAGCGGCCG-3', which destroyed the XbaI site and generated a unique NotI site when ligated into the XbaI site. The ligation mix was used to transform MC1061 cells. Ampicillin-resistant colonies were screened by analysis of NotI-digested colony DNA. The correct plasmid was called pDL323.

To generate vectors pDR601 and pDR602, the *Pichia* URA3 gene was inserted into pDL323 as follows. Plasmid pPU205 (see Figure 9) was digested with PvuII and AatI to liberate the URA3 gene on an approximately 2.5 kb PvuII fragment. The digest was separated on a 0.8% agarose gel. The ~2.5 kb fragment was isolated from the gel using DE81 paper, eluted and purified. Plasmid pDL323

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was linearized by cutting it with EcoRV. This linearized plasmid (~10 ng) was ligated with the *URA3*-bearing PvuII fragment of pPU205 to generate pDR601 and pDR602 (see Figures 14 and 15, respectively), depending upon the orientation of the inserted *URA3* gene.

2. Transformation of IGF-U with pDR601 and pDR602

The *URA3* IGF-1-expressing *P. pastoris* strain IGF-U (see Example VI.A.2.a.) was transformed with linear fragments of DNA derived from pDR601 and pDR602. The linear fragments contained the *URA3* gene flanked on each side with DNA coding for a portion of the *PEP4* gene. Homology between the ends of the fragments and the *PEP4* gene stimulated integration of the fragments at the *PEP4* locus resulting in a gene replacement event. Stable integration of either fragment into the host genome yielded prototrophic transformants due to the stable presence of the *URA3* gene contained in the fragments. The transformation was conducted as follows:

Linear DNA fragments (~4.0 kb in length), consisting of the *URA3* gene flanked on each side with DNA coding for a portion of the *PEP4* gene, were obtained by digesting both pDR601 and pDR602 with NotI and BstEII. The digested DNA (20 µg) was used to transform strain IGF-U using the standard spheroplast procedure. *Ura*⁺ colonies isolated from transformants growing on regeneration medium and subcultured onto YEPD medium were screened for carboxypeptidase Y activity using the overlay procedure described in Example II. Colonies that did not develop a red color relative to control colonies were selected for analysis by Southern blot hybridization.

3. Southern blot hybridization of DNA from transformants

Genomic DNA was isolated from the selected transformants using the method of Hoffman and Winston [Gene 57: 267-272 (1987)]. Genomic DNA from each strain

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was digested with BstEII. This procedure liberates a portion of the *PEP4* locus containing the region of integration of fragments of pDR601 or pDR602. Therefore, the size of this region is diagnostic for correct
5 integration of the transforming DNA into the genome of IGF-U. The digested DNA was subjected to electrophoresis on a 0.8% agarose gel and blotted to a nitrocellulose filter. The filter was hybridized with a radiolabeled 1.4 kb XbaI/EcoRV fragment of pEP301 which contains part
10 of the *P. pastoris PEP4* gene using standard procedures [Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning, A Laboratory Manual, pp 385-388, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA. (1982)]. Hybridization was conducted at 37°C in a
15 solution containing 50% formamide, 6 X SSC, 5X Denhardt's solution, 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 µg/ml salmon sperm DNA. The filter was then washed three times in 1 x SSC, 0.1% SDS (10 min per wash) and then in 0.5 x SSC, 0.1% SDS at 65°C for 1 hr. As a
20 comparative control, genomic DNA from *P. pastoris* strain GS115, a *PEP4* strain, was included in this analysis.

Digestion of genomic DNA from GS115 with BstEII yielded a 4.4 kb fragment that hybridized to the portion of the *PEP4* gene contained in the probe. In contrast,
25 this probe hybridized to a 6.9 kb fragment in DNA from at least two of the transformants, IGFU2601-5 and IGFU2602-5. The larger size of the transformant *PEP4* locus as compared to the control *PEP4* locus (6.9 vs. 4.4 kb) is consistent with replacement of the host *PEP4* gene with a
30 nonfunctional *PEP4* gene carrying the *URA3* gene within its structural region.

From these results, it was concluded that strains IGFU2601-5 and IGFU2602-5 were examples of the several *PEP4* strains generated by disruption of the *PEP4* gene of
35 host strain IGF-U through gene replacement.

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EXAMPLE VII: GENERATION OF A PEP4 PICHIA STRAIN USING "POPOUT" VECTORS**1. Construction of P. pastoris gene disruption vector pDL521**

5 Vector pDL521 was used in the development of *PEP4*-deficient (*PEP4*⁻) strains of *P. pastoris* by disruption of a host *PEP4* gene through "pop-in/pop-out" methods. In this method, a defective *PEP4* gene containing a small deletion is added to a host *PEP4* locus, and a functional
10 *PEP4* gene is removed from the *PEP4* locus (i.e., pop-in/pop-out).

pDL521 was constructed in two steps. First, an intermediate plasmid, pDL501, was constructed by ligation of the 2.2 kb EcoRI/SacI fragment of pDL323, the 2.2 kb
15 SacI/PstI fragment of pPU205 and the 2.7 kb EcoRI/PstI fragment of pUC19 in a three-way ligation. These three fragments were obtained as follows. pPU205, which contains the *P. pastoris* *URA3* gene (Figure 9), was digested with PstI and SacI. A 2.2 kb PstI-SacI fragment
20 containing the *URA3* gene was gel isolated and purified using DE81 paper. Plasmid pDL323, harboring a defective *PEP4* gene which lacks a 0.5 kb *NcoI* fragment present in an intact *PEP4* gene (see Fig. 13), was digested with EcoRI and SacI. A 2.2 kb fragment containing the
25 defective *PEP4* gene was gel isolated and purified using DE81 paper. pUC19 was digested with EcoRI and PstI. The three fragments (0.02 µg of the EcoRI/PstI-digested pUC19, 0.02 µg of the 2.2 kb PstI/SacI fragment of pPU205 and 0.02 µg of the 2.2 kb EcoRI/SacI fragment of pDL323)
30 were ligated in a three-way ligation. The ligation mix was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were screened by analysis of NcoI-digested colony DNA. Plasmid containing the correctly ligated fragments was called pDL501. pDL501 was then cut
35 with SacI, treated with calf alkaline phosphatase and 0.02 µg were ligated with 0.02 µg of a 1.9 kb SacI

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fragment isolated from SacI-digested pEP202 and purified using DE81 paper. This added more *PEP4* flanking sequence to the 3' end of the defective *PEP4* gene in pDL501 and ensured a greater amount of homologous sequence for recombination with the endogenous *PEP4* gene during transformation of *P. pastoris* host IGF-U. The ligation mix was used to transform *E. coli* strain MC1061. DNA from ampicillin-resistant colonies was digested with BglII and SpeI and screened for the presence of the diagnostic 0.8 kb fragment indicative of the presence of the added SacI fragment from pEP202. Correct plasmid was called pDL521 (see Figure 16).

2. Transformation of GS4-2 with pDL521

a. Generation of GS4-2

A *URA3* strain of *P. pastoris* was required as a host in the generation of a *PEP4* strain by the pop-out process. A *URA3* strain was developed by direct plating of 10^6 cells of the general *HIS4* *P. pastoris* host strain GS115 in 5-fluoroorotic acid medium supplemented with uracil (0.67% yeast nitrogen base, 2% agar, 2% glucose, 750 ng 5-FOA/1 and 48 mg uracil/1). After one week of incubation at 30°C, a colony growing on the plate was isolated. This His⁻Ura⁺ strain was named GS4-2.

b. Transformation of GS4-2 and generation of a *PEP4* strain

Plasmid pDL521 was linearized by digestion with NotI. The NotI site is located immediately 5' of the site at which sequence had been deleted from the *PEP4* gene to make it defective. The ends of the NotI fragment are homologous to sequences in the endogenous *PEP4* gene of GS4-2, which promotes integration of the fragment by homologous recombination at the *PEP4* locus.

The His⁻ Ura⁺ strain GS4-2 was transformed according to the spheroplast method with 20 µg of pDL521 which had been linearized by digestion with NotI. Transformants were selected by their ability to grow on media lacking

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uracil. Twelve of these transformants were picked, genomic DNA isolated from these transformants (as described in Example VI.B.3.), cut with SalI and subjected to electrophoresis on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter and probed with a radiolabeled 1.2 kb EcoRV/XbaI fragment of the *PEP4* gene. Two strains, GS4-2521-3 and GS4-2521-4, which appeared to have integrated pDL521 into the *PEP4* locus, based on the Southern blot hybridization pattern of genomic DNA, were chosen for further selection. These strains contained the *URA3* marker gene with an intact complete *PEP4* gene on one side and a defective *PEP4* gene (lacking ~0.5 kb of sequence) on the other side of the marker gene. This configuration of the *PEP4* locus permits recombination between the two copies of the *PEP4* gene that would result in elimination of one of the *PEP4* genes and the *URA3* gene (i.e., pop-out). Either one of the two *PEP4* genes could be evicted in this recombination event. To identify if, and when, recombination between the two *PEP4* genes occurred, strains GS4-2521-3 and GS4-2521-4 were plated onto YPD medium containing 5-FOA in a serial 10-fold dilution manner. Only Ura⁻ strains grow in the presence of 5-FOA, and thus growth in such medium indicates the occurrence of the desired recombination event. Strains able to grow on 5-FOA-containing medium were uracil auxotrophs generated by recombination between the two copies of the *PEP4* gene. Ura⁻ colonies appeared on the 5-FOA-containing plate after 1 week of culture at 30°C: 10 of these colonies were derived from GS4-2521-3, and 14 of these colonies were derived from GS4-2521-4.

3. Characterization of transformants

Fourteen of the Ura⁻ transformant colonies were purified, genomic DNA was prepared from each, digested with EcoRI and EcoRV, subjected to electrophoresis on a

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0.8% agarose gel, blotted to nitrocellulose and hybridized with a radiolabeled 1.2 kb XbaI/EcoRV fragment of the *P. pastoris* *PEP4* gene. DNA from 7 of the 14 isolates analyzed in this way had a hybridization profile consistent with a *PEP4* locus consisting of only a defective *PEP4* gene lacking ~0.5 kb of sequence present in an intact *PEP4* gene. Two of these strains are GS4-2521-3/7 and GS4-2521-4/1.

10 **EXAMPLE VIII: CLONING OF A PORTION OF THE PRB-1 GENE OF P. PASTORIS**

The proteinase B gene, *PRB-1*, encodes a vacuolar serine endoprotease in *S. cerevisiae* [Moehle et al., Mol. Cell Bio. 7: 4390-4399 (1987)]. A portion of the equivalent gene was cloned from *P. pastoris* using polymerase chain reaction (PCR) gene amplification techniques [see, for example, Gould et al., in Proc. Natl. Acad. Sci. USA 86: 1934-1938 (1989)]. Degenerate oligonucleotides, which had homology to sequences of the *PRB-1* gene that encode regions of the proteinase B protein which are conserved across species (Moehle et al. supra.) were synthesized for use as primers in the PCR amplification of *P. pastoris* *PRB-1* DNA. The oligonucleotides had the following sequences:

Oligonucleotide 1:

25 5'- GATAGAATTCTGCAG GGT AAT GGT CAT GGT ACT CAT TGT GC-3'
 A A A G
 C C C C C C C

Oligonucleotide 2:

30 5'- GATCGCATGC AAT CCT GCA ACA TGT GGA GAT GCC AT-3'
 GA A A A
 G G G G G G CTG

To facilitate subcloning of the amplified DNA fragments into shuttle plasmids, each oligonucleotide also contained one or more restriction endonuclease recognition sites on its 5' end: a SphI site on oligonucleotide 2 and both PstI and EcoRI sites on oligonucleotide 1.

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The PCR reaction medium consisted of 100 ng of *P. pastoris* (Strain NRRL Y-11430) genomic DNA in 2 μ l of T.E. (10 mM Tris·HCl, 1 mM EDTA), 10 μ l of oligonucleotide 1 and 10 μ l of oligonucleotide 2, 16 μ l of a 1.25 mM solution of dGTP, dCTP, dATP, and dTTP, 10 μ l of 10x buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.3, 15 mM MgCl₂), 0.1% gelatin, 70 μ l of water and 0.5 μ l of 5 units/ μ l Tag DNA polymerase. The solution was heated at 94°C for 2 minutes. The PCR cycling reaction, which was repeated 31 times, included denaturing for 2 minutes at 96°C, annealing for 1 minute at 50°C and polymerizing for 3.5 minutes at 72°C.

The product of this PCR was subjected to electrophoresis on an agarose gel, and a fragment of the size predicted (~500 bp) for the product of amplification of the PRB-1 gene between positions corresponding to oligonucleotides 1 and 2 was isolated on DE81 paper, and digested with EcoRI and SphI, subjected to electrophoresis on an agarose gel. The 500 bp fragment was isolated using DE81 paper and was ligated into 10 ng of pUC19, which had been linearized by cutting with EcoRI and SphI in the polylinker. The ligation mix was used to transform *E. coli* MC1061 cells. Restriction enzyme-digested plasmid DNA from ampicillin-resistant transformants was analyzed for the presence of the correct 500 bp EcoRI-SphI fragment. One colony contained the correct plasmid, designated pPRBPP. A restriction map of the *Pichia* portion of this plasmid is set forth in Figure 17.

The sequence of the cloned portion of the *P. pastoris* PRB-1 gene contained in pPRBPP was generated using the Sanger dideoxy method (see Sanger et al., supra) and is shown in Sequence ID No. 5. This sequence of the *P. pastoris* PRB-1 gene has approximately 74% homology to the sequence of the *S. cerevisiae* PRB-1 gene.

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EXAMPLE IX: DEVELOPMENT OF A PRB-1 STRAIN OF P. PASTORIS

Plasmid pDR911 was constructed for use in developing PRB-1 strains of *P. pastoris*. This vector contains an internal portion of the *P. pastoris* PRB-1 gene, which, when used to transform PRB-1 strains of *P. pastoris*, integrates into the host genome at the PRB-1 locus to generate two incomplete and non-functional copies of the PRB-1 gene. Vector pDR911 also contains a complete functional *P. pastoris* URA3 gene for use as a selectable marker in URA3 host strains of *P. pastoris*.

A. Construction of pDR911

The PRB-1 gene fragment of *P. pastoris* in pPRBPP was isolated by restriction digestion of pPRBPP with PstI and SphI. The reaction mixture was loaded onto a 0.8% agarose gel and the 0.5 kb fragment was purified with DE81 paper.

This 0.5 kb fragment was ligated into a linear form of plasmid pPU203, a *P. pastoris* URA3-containing pUC-based plasmid (see Figure 8). Plasmid pPU203 was linearized by cleavage with SphI and PstI, and ~10 ng was ligated with ~100 ng of the *Pichia* DNA fragment. The ligation mixture was used to transform *E. coli* strain MC1061 to ampicillin resistance. Ampicillin-resistant colonies were screened by analysis of PstI/SphI-digested colony DNA for the diagnostic fragment. Correct plasmid was named pDR911 (see Figure 18).

B. Transformation of GS4-2 with pDR911

To generate PRB-1 strains of *P. pastoris*, one could transform GS4-2 by standard spheroplast transformation with pDR911 that had been linearized by digestion with BglII. Southern blot hybridization of DNA from Ura⁺ transformants would enable confirmation of PRB-1 strains created by disruption of the PRB-1 locus. Proteinase B activity assays [see, for example, Jones et al., in *Genetics* 102: 665-677 (1982)] of transformants would

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further confirm the proteinase B deficiency of the strains.

C. Development of a prb-1, pep4 strain of *P. pastoris*

5 The PRB-1 gene of the pep4, ura3, his4 strain of *P. pastoris* GS4-2521-4-5, which is an isolate of GS4-2521-4 (see Example VII), was disrupted by transformation with the vector pDR911, which had been linearized by cleavage with BglIII. Transformants exhibiting the Ura⁺3 phenotype
10 were selected and analyzed by Southern blot hybridization. A selected transformant exhibiting the expected hybridization band pattern was designated MG18. This strain was used as a host for expression of IGF-1. The IGF-1 expressing strain was designated C+IGF816S1.

15

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is
20 described and claimed.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Gleeson, Martin A
Howard, Bradley D
- (ii) TITLE OF INVENTION: GENES WHICH INFLUENCE PICHIA PROTEOLYTIC
ACTIVITY, AND USES THEREFOR
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2032 base pairs
 - (B) TYPE: nucleic acid

-67-

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 239..1468

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 239..1468

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCATAA TGGTGAGATT AGGTAATCGT CCGGAATAGG AATAGTGGTT TGGGGCGATT	60
AATCGCACCT GCCTTATATG GTAAGTACCT TGACCGATAA GGTGGCAACT ATTTAGAACA	120
AAGCAAGCCA CCTTTCTTTA TCTGTAAC TC TGTCGAAGCA AGCATCTTTA CTAGAGAACA	180
TCTAAACCAT TTTACATTCT AGAGTTCCAT TTCTCAATTA CTGATAATCA ATTTAAAG	238
ATG ATA TTT GAC GGT ACT ACG ATG TCA ATT GCC ATT GGT TTG CTC TCT	286
Met Ile Phe Asp Gly Thr Thr Met Ser Ile Ala Ile Gly Leu Leu Ser	
1 5 10 15	
ACT CTA GGT ATT GGT GCT GAA GCC AAA GTT CAT TCT GCT AAG ATA CAC	334
Thr Leu Gly Ile Gly Ala Glu Ala Lys Val His Ser Ala Lys Ile His	
20 25 30	
AAG CAT CCA GTC TCA GAA ACT TTA AAA GAG GCC AAT TTT GGG CAG TAT	382
Lys His Pro Val Ser Glu Thr Leu Lys Glu Ala Asn Phe Gly Gln Tyr	
35 40 45	
GTC TCT GCT CTG GAA CAT AAA TAT GTT TCT CTG TTC AAC GAA CAA AAT	430
Val Ser Ala Leu Glu His Lys Tyr Val Ser Leu Phe Asn Glu Gln Asn	
50 55 60	
GCT TTG TCC AAG TCG AAT TTT ATG TCT CAG CAA GAT GGT TTT GCC GTT	478
Ala Leu Ser Lys Ser Asn Phe Met Ser Gln Gln Asp Gly Phe Ala Val	
65 70 75 80	
GAA GCT TCG CAT GAT GCT CCA CTT ACA AAC TAT CTT AAC GCT CAG TAT	526
Glu Ala Ser His Asp Ala Pro Leu Thr Asn Tyr Leu Asn Ala Gln Tyr	
85 90 95	
TTT ACT GAG GTA TCA TTA GGT ACC CCT CCA CAA TCG TTC AAG GTG ATT	574
Phe Thr Glu Val Ser Leu Gly Thr Pro Pro Gln Ser Phe Lys Val Ile	
100 105 110	

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CTT	GAC	ACA	GGA	TCC	TCC	AAT	TTA	TGG	GTT	CCT	AGC	AAA	GAT	TGT	GGA	622
Leu	Asp	Thr	Gly	Ser	Ser	Asn	Leu	Trp	Val	Pro	Ser	Lys	Asp	Cys	Gly	
		115					120					125				
TCA	TTA	GCT	TGC	TTC	TTG	CAT	GCT	AAG	TAT	GAC	CAT	GAT	GAG	TCT	TCT	670
Ser	Leu	Ala	Cys	Phe	Leu	His	Ala	Lys	Tyr	Asp	His	Asp	Glu	Ser	Ser	
		130				135					140					
ACT	TAT	AAG	AAG	AAT	GGT	AGT	AGC	TTT	GAA	ATT	AGG	TAT	GGA	TCC	GGT	718
Thr	Tyr	Lys	Lys	Asn	Gly	Ser	Ser	Phe	Glu	Ile	Arg	Tyr	Gly	Ser	Gly	
145					150					155					160	
TCC	ATG	GAA	GGG	TAT	GTT	TCT	CAG	GAT	GTG	TTG	CAA	ATT	GGG	GAT	TTG	766
Ser	Met	Glu	Gly	Tyr	Val	Ser	Gln	Asp	Val	Leu	Gln	Ile	Gly	Asp	Leu	
				165					170					175		
ACC	ATT	CCC	AAA	GTT	GAT	TTT	GCT	GAG	GCC	ACA	TCG	GAG	CCG	GGG	TTG	814
Thr	Ile	Pro	Lys	Val	Asp	Phe	Ala	Glu	Ala	Thr	Ser	Glu	Pro	Gly	Leu	
			180					185					190			
GCC	TTC	GCT	TTT	GGC	AAA	TTT	GAC	GGA	ATT	TTG	GGG	CTT	GCT	TAT	GAT	862
Ala	Phe	Ala	Phe	Gly	Lys	Phe	Asp	Gly	Ile	Leu	Gly	Leu	Ala	Tyr	Asp	
		195					200					205				
TCA	ATA	TCA	GTA	AAT	AAG	ATT	GTT	CCT	CCA	ATT	TAC	AAG	GCT	TTG	GAA	910
Ser	Ile	Ser	Val	Asn	Lys	Ile	Val	Pro	Pro	Ile	Tyr	Lys	Ala	Leu	Glu	
		210				215					220					
TTA	GAT	CTC	CTT	GAC	GAA	CCA	AAA	TTT	GCC	TTC	TAC	TTG	GGG	GAT	ACG	958
Leu	Asp	Leu	Leu	Asp	Glu	Pro	Lys	Phe	Ala	Phe	Tyr	Leu	Gly	Asp	Thr	
225					230					235					240	
GAC	AAA	GAT	GAA	TCC	GAT	GGC	GGT	TTG	GCC	ACA	TTT	GGT	GGT	GTG	GAC	1006
Asp	Lys	Asp	Glu	Ser	Asp	Gly	Gly	Leu	Ala	Thr	Phe	Gly	Gly	Val	Asp	
				245					250					255		
AAA	TCT	AAG	TAT	GAA	GGA	AAG	ATC	ACC	TGG	TTG	CCT	GTC	AGA	AGA	AAG	1054
Lys	Ser	Lys	Tyr	Glu	Gly	Lys	Ile	Thr	Trp	Leu	Pro	Val	Arg	Arg	Lys	
			260					265					270			
GCT	TAC	TGG	GAG	GTC	TCT	TTT	GAT	GGT	GTA	GGT	TTG	GGA	TCC	GAA	TAT	1102
Ala	Tyr	Trp	Glu	Val	Ser	Phe	Asp	Gly	Val	Gly	Leu	Gly	Ser	Glu	Tyr	
		275					280					285				
GCT	GAA	TTG	CAA	AAA	ACT	GGT	GCA	GCC	ATC	GAC	ACT	GGA	ACC	TCA	TTG	1150
Ala	Glu	Leu	Gln	Lys	Thr	Gly	Ala	Ala	Ile	Asp	Thr	Gly	Thr	Ser	Leu	
		290				295					300					

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ATT GCT TTG CCC AGT GGC CTA GCT GAA ATT CTC AAT GCA GAA ATT GGT Ile Ala Leu Pro Ser Gly Leu Ala Glu Ile Leu Asn Ala Glu Ile Gly 305 310 315 320	1198
GCT ACC AAG GGT TGG TCT GGT CAA TAC GCT GTG GAC TGT GAC ACT AGA Ala Thr Lys Gly Trp Ser Gly Gln Tyr Ala Val Asp Cys Asp Thr Arg 325 330 335	1246
GAC TCT TTG CCA GAC TTA ACT TTA ACC TTC GCC GGT TAC AAC TTT ACC Asp Ser Leu Pro Asp Leu Thr Leu Thr Phe Ala Gly Tyr Asn Phe Thr 340 345 350	1294
ATT ACT CCA TAT GAC TAT ACT TTG GAG GTT TCT GGG TCA TGT ATT AGT Ile Thr Pro Tyr Asp Tyr Thr Leu Glu Val Ser Gly Ser Cys Ile Ser 355 360 365	1342
GCT TTC ACC CCC ATG GAC TTT CCT GAA CCA ATA GGT CCT TTG GCA ATC Ala Phe Thr Pro Met Asp Phe Pro Glu Pro Ile Gly Pro Leu Ala Ile 370 375 380	1390
ATT GGT GAC TCG TTC TTG AGA AAA TAT TAC TCA GTT TAT GAC CTA GGC Ile Gly Asp Ser Phe Leu Arg Lys Tyr Tyr Ser Val Tyr Asp Leu Gly 385 390 395 400	1438
AAA GAT GCA GTA GGT TTA GCC AAG TCT ATT TAGGCAAGAA TAAAAGTTGC Lys Asp Ala Val Gly Leu Ala Lys Ser Ile 405 410	1488
TCAGCTGAAC TTATTTGGTT ACTTATCAGG TAGTGAAGAT GTAGAGAATA TATGTTTAGG	1548
TATTTTTTTT TAGTTTTTCT CCTATAACTC ATCTTCAGTA CGTGATTGCT TGTCAGCTAC	1608
CTTGACAGGG GCGCATAAGT GATATCGTGT ACTGCTCAAT CAAGATTTGC CTGCTCCATT	1668
GATAAGGGTA TAAGAGACCC ACCTGCTCCT CTTTAAATTT CTCTCTTAAC TGTTGTGAAA	1728
ATCATCTTCG AAGCAAATTC GAGTTTAAAT CTATGCGGTT GGTAAGTAAA GGTATGTCAT	1788
GGTGGTATAT AGTTTTTCAT TTTACCTTTT ACTAATCAGT TTTACAGAAG AGGAACGTCT	1848
TTCTCAAGAT CGAAATAGGA CTAAATACTG GAGACGATGG GGTCCATTATT TGGGTGAAAG	1908
GCAGTGGGCT ACAGTAAGGG AAGACTATTC CGATGATGGA GATGCTTGGT CTGCTTTTCC	1968
TTTTGAGCAA TCTCATTGA GAACTTATCG CTGGGGAGAG GATGGACTAG CTGGAGTCTC	2028
AGAC	2032

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 410 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ile Phe Asp Gly Thr Thr Met Ser Ile Ala Ile Gly Leu Leu Ser
 1             5             10             15

Thr Leu Gly Ile Gly Ala Glu Ala Lys Val His Ser Ala Lys Ile His
      20             25             30

Lys His Pro Val Ser Glu Thr Leu Lys Glu Ala Asn Phe Gly Gln Tyr
      35             40             45

Val Ser Ala Leu Glu His Lys Tyr Val Ser Leu Phe Asn Glu Gln Asn
      50             55             60

Ala Leu Ser Lys Ser Asn Phe Met Ser Gln Gln Asp Gly Phe Ala Val
      65             70             75             80

Glu Ala Ser His Asp Ala Pro Leu Thr Asn Tyr Leu Asn Ala Gln Tyr
      85             90             95

Phe Thr Glu Val Ser Leu Gly Thr Pro Pro Gln Ser Phe Lys Val Ile
      100            105            110

Leu Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Lys Asp Cys Gly
      115            120            125

Ser Leu Ala Cys Phe Leu His Ala Lys Tyr Asp His Asp Glu Ser Ser
      130            135            140

Thr Tyr Lys Lys Asn Gly Ser Ser Phe Glu Ile Arg Tyr Gly Ser Gly
      145            150            155            160

Ser Met Glu Gly Tyr Val Ser Gln Asp Val Leu Gln Ile Gly Asp Leu
      165            170            175

Thr Ile Pro Lys Val Asp Phe Ala Glu Ala Thr Ser Glu Pro Gly Leu
      180            185            190

Ala Phe Ala Phe Gly Lys Phe Asp Gly Ile Leu Gly Leu Ala Tyr Asp
      195            200            205

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Ser Ile Ser Val Asn Lys Ile Val Pro Pro Ile Tyr Lys Ala Leu Glu
 210 215 220
 Leu Asp Leu Leu Asp Glu Pro Lys Phe Ala Phe Tyr Leu Gly Asp Thr
 225 230 235 240
 Asp Lys Asp Glu Ser Asp Gly Gly Leu Ala Thr Phe Gly Gly Val Asp
 245 250 255
 Lys Ser Lys Tyr Glu Gly Lys Ile Thr Trp Leu Pro Val Arg Arg Lys
 260 265 270
 Ala Tyr Trp Glu Val Ser Phe Asp Gly Val Gly Leu Gly Ser Glu Tyr
 275 280 285
 Ala Glu Leu Gln Lys Thr Gly Ala Ala Ile Asp Thr Gly Thr Ser Leu
 290 295 300
 Ile Ala Leu Pro Ser Gly Leu Ala Glu Ile Leu Asn Ala Glu Ile Gly
 305 310 315 320
 Ala Thr Lys Gly Trp Ser Gly Gln Tyr Ala Val Asp Cys Asp Thr Arg
 325 330 335
 Asp Ser Leu Pro Asp Leu Thr Leu Thr Phe Ala Gly Tyr Asn Phe Thr
 340 345 350
 Ile Thr Pro Tyr Asp Tyr Thr Leu Glu Val Ser Gly Ser Cys Ile Ser
 355 360 365
 Ala Phe Thr Pro Met Asp Phe Pro Glu Pro Ile Gly Pro Leu Ala Ile
 370 375 380
 Ile Gly Asp Ser Phe Leu Arg Lys Tyr Tyr Ser Val Tyr Asp Leu Gly
 385 390 395 400
 Lys Asp Ala Val Gly Leu Ala Lys Ser Ile
 405 410

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2688 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 643..1431

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 643..1431

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGCAGAAAT GGGGAGATAA CCACCTTTGA CGAATTGACT AAAGTTCTAC AGATCATGTT	60
TACAAATGCC ATCATCTATA ACGATGAAGA CAGTGATGTT TCGAAGCTAA CGATTGAAAT	120
GATGGAAGAA ACTACTAAGA TTATAGAGCT GTTCAGAGAA AGTCTGGATT AGTCCTGGAC	180
AATGAACTTT ATGTACAAAA ATATGGGGTT AACGTCTTAG CTGTTGCATC ATAAGTTGGT	240
TTTGTTCCTG GAAACGTTGA CCAACTCTCT CACTGTGCTT GAGGAACTTT TCTGCACACT	300
TGTTGATGCA GCCTTCCTCC TTAGAAGTCA ACTTGTTAGA TGAAAAATCA TTGACACAGT	360
CTGTAAAACA TTTGCTAACC AAATCGGAGT AAAGACGCAT GAAGTCTTTC ATTTGTTTTT	420
GTTCAACGAG TTTCTGGAAC TCTTGTTGTT CTTTAGCGTT CAATGCGTCC ATTTTGTGAT	480
GTACTTGATT GGGGTAGAGT TAGCACTTGC TCTCTCTGTT ACCAGTTTTT GTCAAGATTG	540
AAGAAAAAAG TTTTTTGGAC GGTACACGTC GCACCTATCC TTCGCATTGA TCCACTCTAA	600
TGAGTTAACA TCAACCTGAT CAAAGGGATA GATACCTAGA CA ATG GCT CGC AGT	654
Met Ala Arg Ser	
1	
TAT GCC GAG AGA GCA AAT ACT CAT CAA TCA CCT GTG GCA CGA CGA CTG	702
Tyr Ala Glu Arg Ala Asn Thr His Gln Ser Pro Val Ala Arg Arg Leu	
5 10 15 20	
TTT GCG CTT ATG GAA CAG AAA CAG AGT AAC CTA TGC GCA TCA GTC GAC	750
Phe Ala Leu Met Glu Gln Lys Gln Ser Asn Leu Cys Ala Ser Val Asp	
25 30 35	
GTG AGA ACA ACT AAA GAA TTA TTG GAG CTT CTA GAT AAA TTG GGC CCA	798
Val Arg Thr Thr Lys Glu Leu Leu Glu Leu Leu Asp Lys Leu Gly Pro	
40 45 50	
TTT ATC TGT TTG GCC AAG ACT CAT ATC GAC ATA ATT GAT GAC TTC ACG	846
Phe Ile Cys Leu Ala Lys Thr His Ile Asp Ile Ile Asp Asp Phe Thr	
55 60 65	

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TAT GAT GGA ACT ATT CTG CCT TTA TTG GAA CTA TCA AAG AAA CAC AAG Tyr Asp Gly Thr Ile Leu Pro Leu Leu Glu Leu Ser Lys Lys His Lys 70 75 80	894
TTT TTA ATT TTT GAG GAC AGA AAG TTT GCT GAT ATA GGC AAC ACT GTC Phe Leu Ile Phe Glu Asp Arg Lys Phe Ala Asp Ile Gly Asn Thr Val 85 90 95 100	942
AAG CAT CAA TAT CAA GGA GGT GTC TAC AAG ATT GCA CAA TGG GCA GAT Lys His Gln Tyr Gln Gly Gly Val Tyr Lys Ile Ala Gln Trp Ala Asp 105 110 115	990
ATT ACA AAT GCT CAT GGT GTC ATT GGT AGT GGA ATT GTA AAG GGT CTA Ile Thr Asn Ala His Gly Val Ile Gly Ser Gly Ile Val Lys Gly Leu 120 125 130	1038
AAG GAG GCA GCC ACT GAG ACA ACA GAT CAA CCA AGG GGA CTA TTG ATG Lys Glu Ala Ala Thr Glu Thr Thr Asp Gln Pro Arg Gly Leu Leu Met 135 140 145	1086
TTG GCT GAA CTG TCG TCA AAG GGA TCA ATT GCC CAT GGT AAG TAC ACC Leu Ala Glu Leu Ser Ser Lys Gly Ser Ile Ala His Gly Lys Tyr Thr 150 155 160	1134
GAA GAA ACT GTA GAA ATT GCA AAA TCA GAC AAG GAA TTC GTC ATT GGC Glu Glu Thr Val Glu Ile Ala Lys Ser Asp Lys Glu Phe Val Ile Gly 165 170 175 180	1182
TTT ATT GCT CAA AAT TCT ATG GGA GGA CAA GAT GAA GGG TTC GAT TGG Phe Ile Ala Gln Asn Ser Met Gly Gly Gln Asp Glu Gly Phe Asp Trp 185 190 195	1230
ATT ATT ATG ACA CCA GGT GTT GGT TTG GAT GAC ACT GGT GAT GCT CTA Ile Ile Met Thr Pro Gly Val Gly Leu Asp Asp Thr Gly Asp Ala Leu 200 205 210	1278
GGC CAA CAA TAT CGA ACA GTG AGT CAA GTA TTT TCC ACT GGC ACT GAC Gly Gln Gln Tyr Arg Thr Val Ser Gln Val Phe Ser Thr Gly Thr Asp 215 220 225	1326
ATC ATA ATC GTA GGT CGT GGT TTG TTT GGC AAG GGC AGA GAT CCC TTA Ile Ile Ile Val Gly Arg Gly Leu Phe Gly Lys Gly Arg Asp Pro Leu 230 235 240	1374
AAA GAA GGT GAA CGG TAT AGA AAA GCT GGG TGG GAA GCT TAC CAA AAT Lys Glu Gly Glu Arg Tyr Arg Lys Ala Gly Trp Glu Ala Tyr Gln Asn 245 250 255 260	1422
ATT CTG AGG TAAATTACAA GTATGTACAG GGGATCAATT GTTTCGGGCG Ile Leu Arg	1471

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ATTCAACTGA ATCGATCTTC AATTTTCATCG CTCAATTTTT GACGCAGTAT TTCAAACACC	1531
AGAAGCCCCA CGGATGTTGC TGAATGGTA GTTAACGCAT TCCTAACGAA CCCTTTATAA	1591
AACCAGCGGG TCCAAGATAG TTTAGACTTC TCATGTAAGC TCACCAACTG GTGGAATGTA	1651
TCTAAGTATG ATCGGTAATA TAGACGGAAT TTACTTTTCT TATCCCAGGA GTTCTCGTTG	1711
AAAATATCCA ACGCTTCCAA CCTTGCTAAA TGTATTGACT GAACTTTAGA AAATGGGTAT	1771
TGAACGGCTA GTAACGAACA TGCAGCGCTA GCACCAGCCA AAAGAATAAA AGTCGTCCTC	1831
AGGATATTTT CACTTTTCGT TTTCCTGTG TCACCTTGGG GCCTTCCAAG AAGACTATTT	1891
TTCATCCTAT CAATTCTCTC CATAGTGTTT TCGGTTATCC TGTAACCTCT ATTCTTAATG	1951
GCTTCGAATG TTGTGAAATA TATAGCAAAG GATGTGCTTT CTTTGACCAG ACTCAAGGAG	2011
TAGCCAGCAA ATACCCCCAG AAAACCACTA GTTTTGTAGT TATGAAGACC GTAAATCCAT	2071
AAGTTGTCAT TCTTGCCCCC AATAATCTCG GAGGCATTAG ATCGGGCATA TATTGCATCA	2131
ATTGGGGCAG CTACCAATGA CTGCGCAGCT CCAGCTAGAA ACCCAGCTCG AAATACATCC	2191
ACTAGTCTTG GATTTGCTAT CGATCTGCCC TCTTGACCGT CAGTATATGA CTGCAAACAT	2251
GATAAATACG TTGTGTAAAG TACAATTCCC ATCACAGAAT TGGCTACCAA TGGTGGCAGG	2311
ACCTTGTTTG GTATCAACTC CCAACCATGG GTTTTGACGG CTCGTAACAA TAGAGCTGGA	2371
TTTGAGTGGA AAATGGGCTG TAAGGTTTAC CTTTCAAATG AGCTCCAAAG AAGATGCGTA	2431
TTGGTGCCAT GTAGTCAAAA CGAGTGGGAC GAAACAGTTT GGCTGGTGTC CTCAGGTACA	2491
GTGAACTAAA TTGGACTAGA ACAGCTCTGA TCCAGCTGT CGAAGCAGAC ACCACTTGAG	2551
TGTTTTTGTT GCTAAGAGTA GCCTTTTGTAG AATCATCGTT GTCTTCATA GGTTCCTGGA	2611
ACACAATGCC AGAGTTCATA GAGGATCAGA GGGGAATTGA GGTGTGTGTA TATGTATTTA	2671
TAGGGGTACC GAGCTCG	2688

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 263 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Ala Arg Ser Tyr Ala Glu Arg Ala Asn Thr His Gln Ser Pro Val
 1           5           10           15

Ala Arg Arg Leu Phe Ala Leu Met Glu Gln Lys Gln Ser Asn Leu Cys
 20           25           30

Ala Ser Val Asp Val Arg Thr Thr Lys Glu Leu Leu Glu Leu Leu Asp
 35           40           45

Lys Leu Gly Pro Phe Ile Cys Leu Ala Lys Thr His Ile Asp Ile Ile
 50           55           60

Asp Asp Phe Thr Tyr Asp Gly Thr Ile Leu Pro Leu Leu Glu Leu Ser
 65           70           75           80

Lys Lys His Lys Phe Leu Ile Phe Glu Asp Arg Lys Phe Ala Asp Ile
 85           90           95

Gly Asn Thr Val Lys His Gln Tyr Gln Gly Gly Val Tyr Lys Ile Ala
100           105           110

Gln Trp Ala Asp Ile Thr Asn Ala His Gly Val Ile Gly Ser Gly Ile
115           120           125

Val Lys Gly Leu Lys Glu Ala Ala Thr Glu Thr Thr Asp Gln Pro Arg
130           135           140

Gly Leu Leu Met Leu Ala Glu Leu Ser Ser Lys Gly Ser Ile Ala His
145           150           155           160

Gly Lys Tyr Thr Glu Glu Thr Val Glu Ile Ala Lys Ser Asp Lys Glu
165           170           175

Phe Val Ile Gly Phe Ile Ala Gln Asn Ser Met Gly Gly Gln Asp Glu
180           185           190

Gly Phe Asp Trp Ile Ile Met Thr Pro Gly Val Gly Leu Asp Asp Thr
195           200           205

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Gly Asp Ala Leu Gly Gln Gln Tyr Arg Thr Val Ser Gln Val Phe Ser
 210 215 220

Thr Gly Thr Asp Ile Ile Ile Val Gly Arg Gly Leu Phe Gly Lys Gly
 225 230 235 240

Arg Asp Pro Leu Lys Glu Gly Glu Arg Tyr Arg Lys Ala Gly Trp Glu
 245 250 255

Ala Tyr Gln Asn Ile Leu Arg
 260

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 555 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..554

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 3..554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GA ATT CTG CAG GGA AAC GGC CAC GGT ACA CAT TGT GCT GGT ACC ATT	47
Ile Leu Gln Gly Asn Gly His Gly Thr His Cys Ala Gly Thr Ile	
1 5 10 15	
GCT TCT GAA AGC TAC GGT GTT GCC AAG AAG GCT AAT GTT GTT GCC ATC	95
Ala Ser Glu Ser Tyr Gly Val Ala Lys Lys Ala Asn Val Val Ala Ile	
20 25 30	
AAG GTC TTG AGA TCT AAT GGT TCT GGT TCG ATG TCA GAT GTT CTG AAG	143
Lys Val Leu Arg Ser Asn Gly Ser Gly Ser Met Ser Asp Val Leu Lys	
35 40 45	
GGT GTT GAG TAT GCC ACC CAA TCC CAC TTG GAT GCT GTT AAA AAG GGC	191
Gly Val Glu Tyr Ala Thr Gln Ser His Leu Asp Ala Val Lys Lys Gly	
50 55 60	

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AAC AAG AAA TTT AAG GGC TCT ACC GCT AAC ATG TCA CTG GGT GGT GGT Asn Lys Lys Phe Lys Gly Ser Thr Ala Asn Met Ser Leu Gly Gly Gly 65 70 75	239
AAA TCT CCT GCT TTG GAC CTT GCA GTC AAT GCT GCT GTT AAG AAT GGT Lys Ser Pro Ala Leu Asp Leu Ala Val Asn Ala Ala Val Lys Asn Gly 80 85 90 95	287
ATT CAC TTT GCC GTT GCA GCA GGT AAC GAA AAC CAA GAT GCT TGT AAC Ile His Phe Ala Val Ala Ala Gly Asn Glu Asn Gln Asp Ala Cys Asn 100 105 110	335
ACC TCG CCA GCA GCT GCT GAG AAT GCC ATC ACC GTC GGT GCA TCA ACC Thr Ser Pro Ala Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser Thr 115 120 125	383
TTA TCA GAC GCT AGA GCT TAC TTT TCT AAC TAC GGT AAA TGT GTT GAC Leu Ser Asp Ala Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Val Asp 130 135 140	431
ATT TTC GCT CCA GGT TTA AAC ATT CTT TCT ACC TAC ACT GGT TCG GAT Ile Phe Ala Pro Gly Leu Asn Ile Leu Ser Thr Tyr Thr Gly Ser Asp 145 150 155	479
GAC GCA ACT GCT ACC TTG TCT GGT ACT TCA ATG GCC AGC CCT CAT GTT Asp Ala Thr Ala Thr Leu Ser Gly Thr Ser Met Ala Ser Pro His Val 160 165 170 175	527
GCA GGC TTG CAT GCA AGC TTG GCA CTG G Ala Gly Leu His Ala Ser Leu Ala Leu 180	555

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 184 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Leu Gln Gly Asn Gly His Gly Thr His Cys Ala Gly Thr Ile Ala 1 5 10 15
Ser Glu Ser Tyr Gly Val Ala Lys Lys Ala Asn Val Val Ala Ile Lys 20 25 30

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Val Leu Arg Ser Asn Gly Ser Gly Ser Met Ser Asp Val Leu Lys Gly
 35 40 45

Val Glu Tyr Ala Thr Gln Ser His Leu Asp Ala Val Lys Lys Gly Asn
 50 55 60

Lys Lys Phe Lys Gly Ser Thr Ala Asn Met Ser Leu Gly Gly Gly Lys
 65 70 75 80

Ser Pro Ala Leu Asp Leu Ala Val Asn Ala Ala Val Lys Asn Gly Ile
 85 90 95

His Phe Ala Val Ala Ala Gly Asn Glu Asn Gln Asp Ala Cys Asn Thr
 100 105 110

Ser Pro Ala Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser Thr Leu
 115 120 125

Ser Asp Ala Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Val Asp Ile
 130 135 140

Phe Ala Pro Gly Leu Asn Ile Leu Ser Thr Tyr Thr Gly Ser Asp Asp
 145 150 155 160

Ala Thr Ala Thr Leu Ser Gly Thr Ser Met Ala Ser Pro His Val Ala
 165 170 175

Gly Leu His Ala Ser Leu Ala Leu
 180

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THAT WHICH IS CLAIMED IS:

1. An isolated DNA fragment obtained from a strain of the genus *Pichia*, comprising a gene encoding a protein which, directly or indirectly, influences the proteolytic activity of said strain.

2. The DNA fragment of Claim 1, wherein said protein influences the carboxypeptidase Y activity of said strain.

3. The DNA fragment of Claim 2, wherein said gene has the restriction map in FIG. 1 of the drawings.

4. The DNA fragment of Claim 2, wherein said fragment is the approximately 10.6 kbp EcoRI fragment of plasmid pEP202.

5. The DNA fragment of Claim 2, wherein said fragment is the approximately 2.7 kbp EcoRI-SacI fragment of plasmid pEP301, or portions thereof that encode a protein which influences the carboxypeptidase Y activity of said strain.

6. The DNA fragment of Claim 2, wherein said the nucleic acid sequence of said gene encodes an amino acid sequence which is substantially the same as the amino acid sequence set forth in Sequence ID No. 2.

7. The DNA fragment of Claim 2, wherein the nucleic acid sequence of said gene is substantially the same as the nucleic acid sequence set forth in Sequence ID No. 1.

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8. An isolated DNA fragment containing a modified form of the gene of Claim 1, wherein said modification renders the gene incapable of producing functional product, or alters the ability of the gene product to influence the proteolytic activity of said strain.

9. The DNA fragment of Claim 8, wherein the modified form of said gene is suitable for introduction into a yeast host of the genus *Pichia* by homologous recombination, wherein homologous recombination occurs at the specific locus of said gene whose expression product influences proteolytic activity.

10. The DNA fragment of Claim 8, wherein the modified gene, in its unmodified form, encodes a protein that influences the carboxypeptidase Y activity of said strain.

11. The DNA fragment of Claim 9, wherein said gene is modified by the insertion of an auxotrophic marker gene therein.

12. A DNA fragment of Claim 11, wherein said auxotrophic marker gene is selected from the *Pichia* or *Saccharomyces HIS4* gene, the *Pichia* or *Saccharomyces ARG4* gene, or the *Pichia* or *Saccharomyces URA3* gene.

13. The DNA fragment of Claim 12, wherein said fragment is included in plasmid pDR401.

14. The DNA fragment of Claim 9, wherein said gene is modified by making deletions therefrom.

15. The DNA fragment of Claim 14, wherein said fragment is included in plasmid pDR421.

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16. A method of producing strain(s) of the genus *Pichia* which are deficient in proteolytic activity, compared to host strain(s) of the same species, comprising:

5 contacting said host strain(s) with the DNA fragment of Claim 8 under conditions suitable for the site-directed integration of said DNA fragment into the genome of said host strain(s), wherein said site-directed integration occurs at the specific locus of said gene
10 which encodes a protein which influences proteolytic activity.

17. The method of Claim 16, further comprising:
screening the strains obtained as a result of said
15 contacting for the presence of strains which have reduced proteolytic activity, relative to said host strain(s), and

selecting those strains which have reduced
proteolytic activity, relative to said host strain(s).
20

18. The method of Claim 16, wherein integration of said DNA fragment influences the proteinase A and carboxypeptidase Y activities of said host organism.

25 19. A strain of *Pichia* deficient in proteolytic activity produced by the method of Claim 16.

20. The strain of Claim 19, which is deficient in proteinase A and carboxypeptidase Y activities.
30

21. The strain of Claim 20 selected from *P. pastoris* strain p1, p2, p5, p8, p13, p16, or p20.

22. The method of Claim 16, wherein said host
35 strain is defective in at least one auxotrophic marker

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gene selected from the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the orotidine-5'-phosphate decarboxylase gene, and wherein the modified gene of said fragment is modified by the insertion
5 therein of an intact form of the auxotrophic marker gene in which the host strain is defective.

10 23. A strain of *Pichia* deficient in proteolytic activity produced by the method of Claim 22.

24. A strain of Claim 23, wherein said strain is deficient in proteinase A and carboxypeptidase Y activity.

15 25. A strain of Claim 24 selected from *P. pastoris* strain p1, p2, p5, p8, p13, p16, or p20.

20 26. The method of Claim 16, wherein the gene said fragment is modified by making deletions therefrom.

27. The method of Claim 22, wherein said host strain is GS115 and said DNA fragment is the approximately 5.3 kbp SacI-EcoRI fragment of plasmid pDR401.

25 28. A yeast cell of the genus *Pichia* which is deficient in proteolytic activity, compared to wild-type strains of the same species.

30 29. The yeast cell of Claim 28, wherein said cell deficient in proteinase A and carboxypeptidase Y activities.

35 30. A method for the expression of proteolytically sensitive recombinant product(s), comprising:

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transforming the cell of Claim 28 with DNA encoding said product and culturing said cell under conditions whereby said proteolytically sensitive product(s) is expressed.

5 31. The method of claim 30, wherein the cell is rendered deficient in proteolytic activity by replacement of a gene of the cell that encodes a protein which, upon expression, directly or indirectly, influences the carboxypeptidase Y activity of said host organism with a
10 defective form of said gene modified by the insertion therein of a marker gene which complements an auxotrophic phenotype of said host strain; and wherein the marker gene is selected from the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the
15 orotidine-5'-phosphate decarboxylase gene.

 32. A method for the expression of proteolytically sensitive recombinant product(s), comprising
 rendering a host strain deficient in proteolytic
20 activity using the method of Claim 16, wherein
 said host strain is defective in at least one auxotrophic marker gene; and
 said host strain has been transformed with at least one DNA fragment comprising an expression cassette
25 comprising, in the reading frame direction of transcription, the following sequences of nucleotides:
 (i) a promoter region of a methanol-responsive gene of a methylotrophic yeast,
 (ii) a sequence encoding a polypeptide consisting
30 essentially of:
 (a) an optional secretion signal sequence, and
 (b) a proteolytically sensitive protein; and
 (iii) a transcription terminator functional in a methylotrophic yeast;

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wherein said sequences are operationally associated with one another for transcription of the sequences encoding said polypeptide.

5 33. The method of Claim 32, wherein said host strain is defective in at least two auxotrophic marker genes.

 34. A method of Claim 33, wherein said auxotrophic
10 marker genes are the *HIS4* gene, and the *URA3* gene.

 35. A method of Claim 34, wherein the
proteolytically sensitive product is IGF-1 and the marker
gene employed for transforming said host with DNA
15 encoding IGF-1 is the *HIS4* gene.

 36. A method of Claim 35, wherein the modified gene
employed to render the host deficient in proteolytic
activity, in its unmodified form, encodes a protein that
20 influences the carboxypeptidase activity of the host.

 37. A method of Claim 36, wherein the modified gene
is produced by deletion of a portion of the coding
sequence therefrom.
25

 38. A method of Claim 37, wherein the recombinant
strain from which said recombinant product(s) is
expressed is strain M+IMB206S1.

30 39. An isolated DNA fragment, comprising the
orotidine-5'-phosphate decarboxylase gene from species of
the genus *Pichia*.

-85-

40. The DNA fragment of Claim 39, wherein said gene has substantially the same restriction map as shown in FIG. 12.

5 41. The DNA fragment of Claim 39, wherein said gene encodes substantially the same amino acid sequence as set forth in Sequence ID No. 4.

10 42. A DNA fragment of Claim 39, wherein said gene has substantially the same nucleic acid sequence as set forth in Sequence ID No. 3.

15 43. A yeast cell of the genus *Pichia*, which is defective in the orotidine-5'-phosphate decarboxylase gene.

44. The yeast cell of Claim 43, wherein said yeast cell is a strain of *Pichia pastoris*.

20 45. The yeast cell of Claim 44, wherein said yeast cell is the strain *Pichia pastoris* GS4-2.

25 46. The yeast cell of Claim 43, further containing a deficiency in proteolytic activity.

47. A yeast cell of Claim 46, wherein said yeast cell is deficient in proteinase A and carboxypeptidase Y activities.

30 48. The yeast cell of Claim 47, wherein said yeast cell is selected from the *Pichia pastoris* strains GS4-2521-3/7 or GS4-2521-4/1.

(Linear) MAPLOT of: Ppap4.Seq ck: 8585, 1 to: 2032 March 18, 1991 12:00.

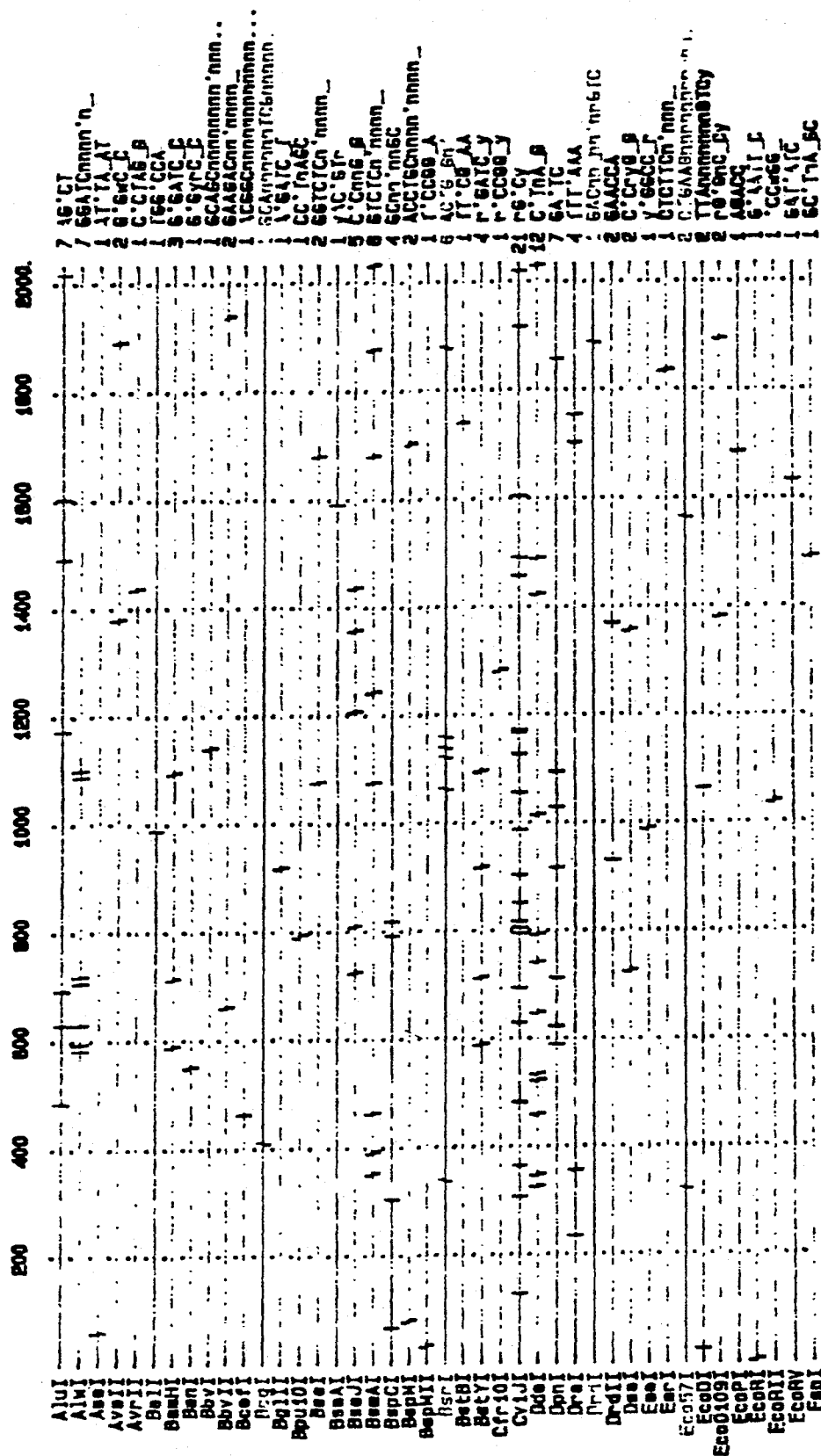


FIG. 1-1

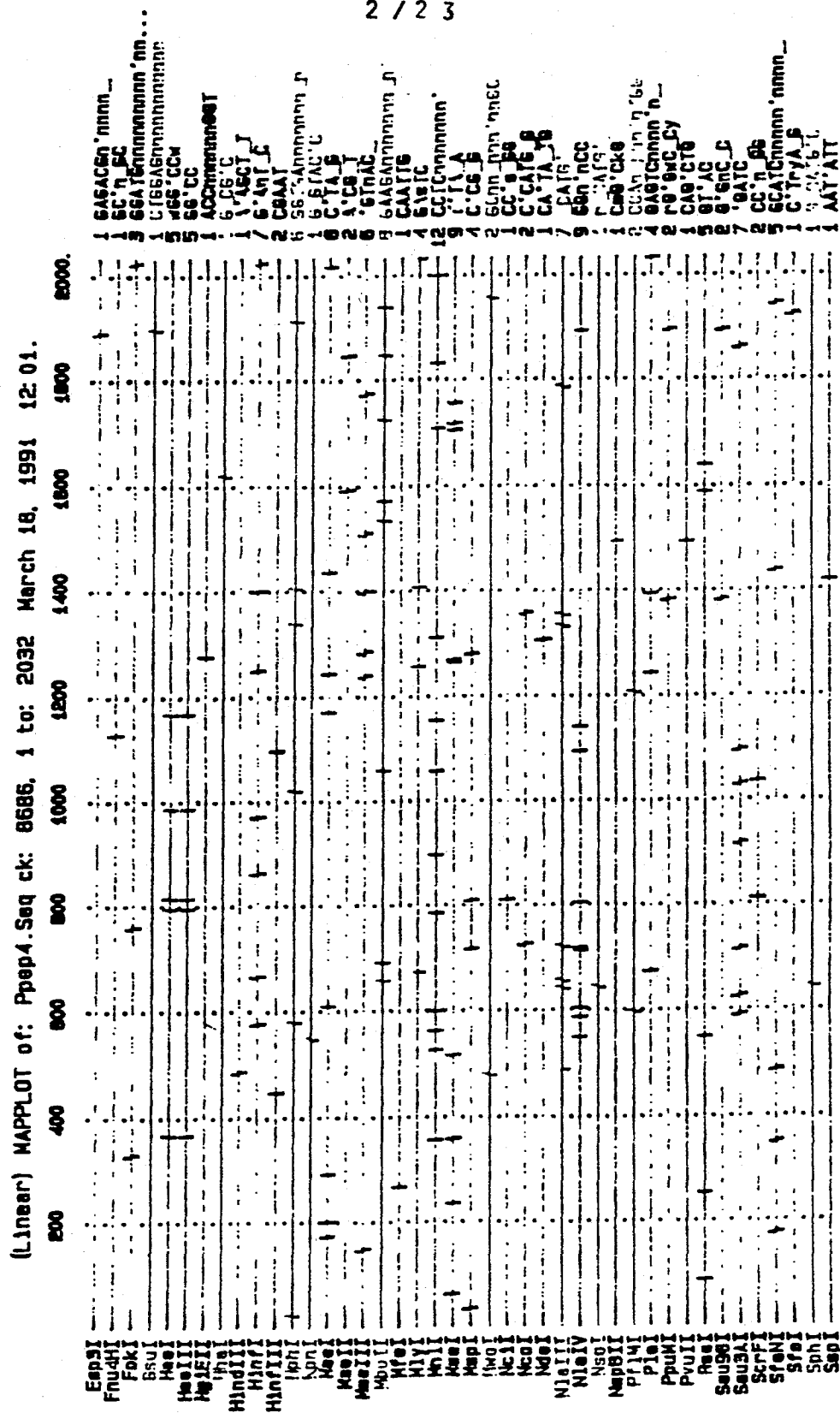


FIG. 1-2

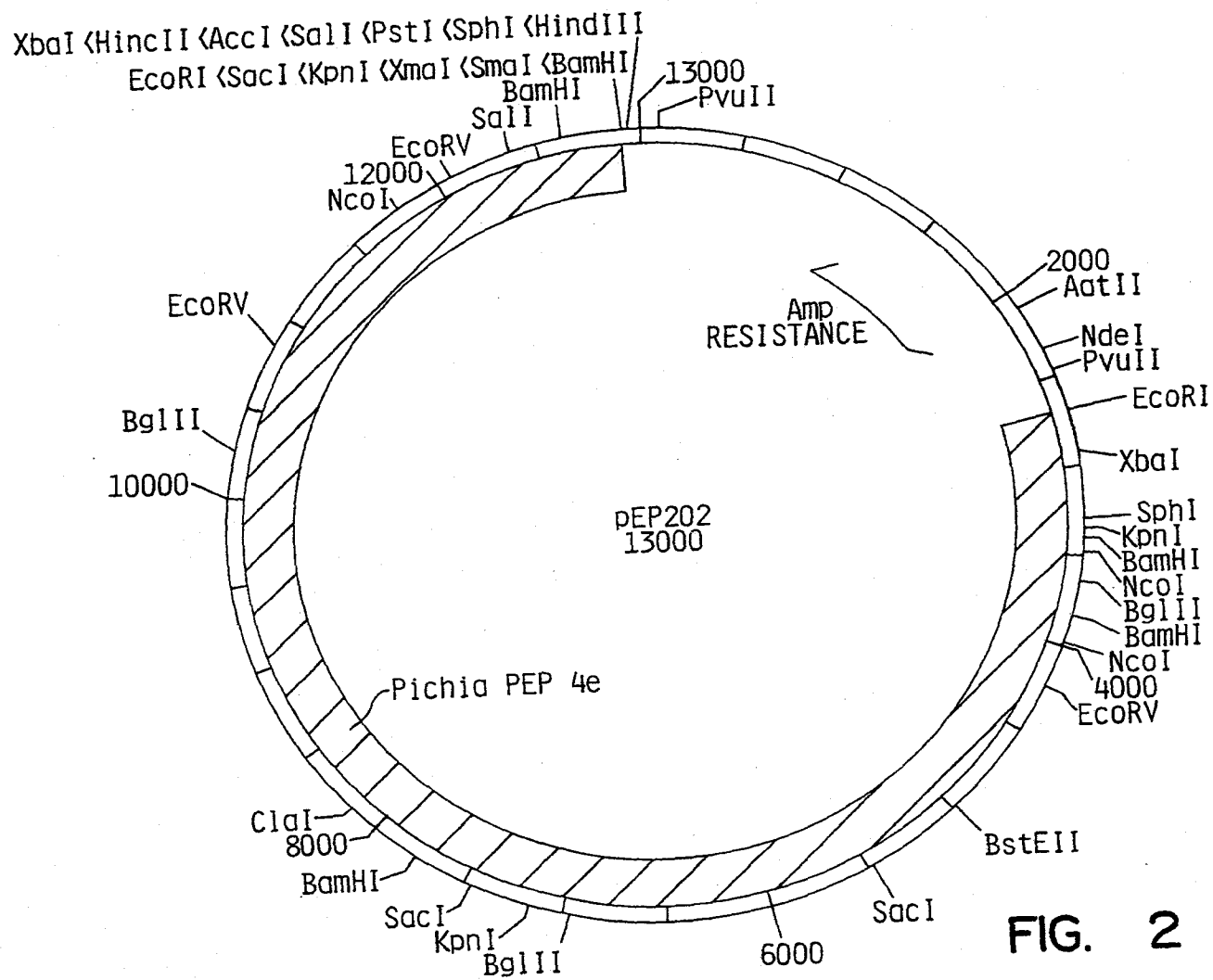
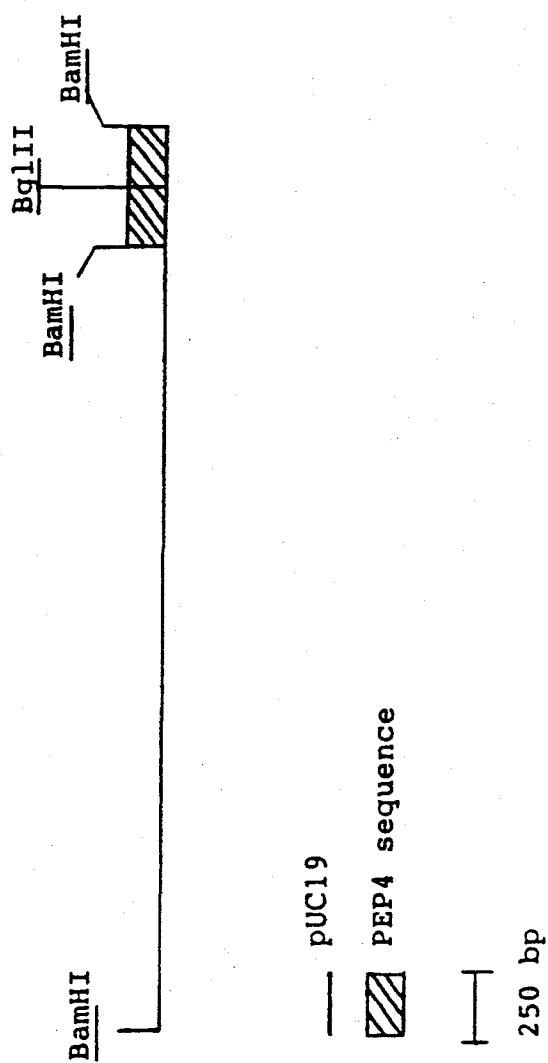


FIG. 2

5 / 2 3

FIGURE 3
Linear Restriction Map of pEP205



6 / 2 3

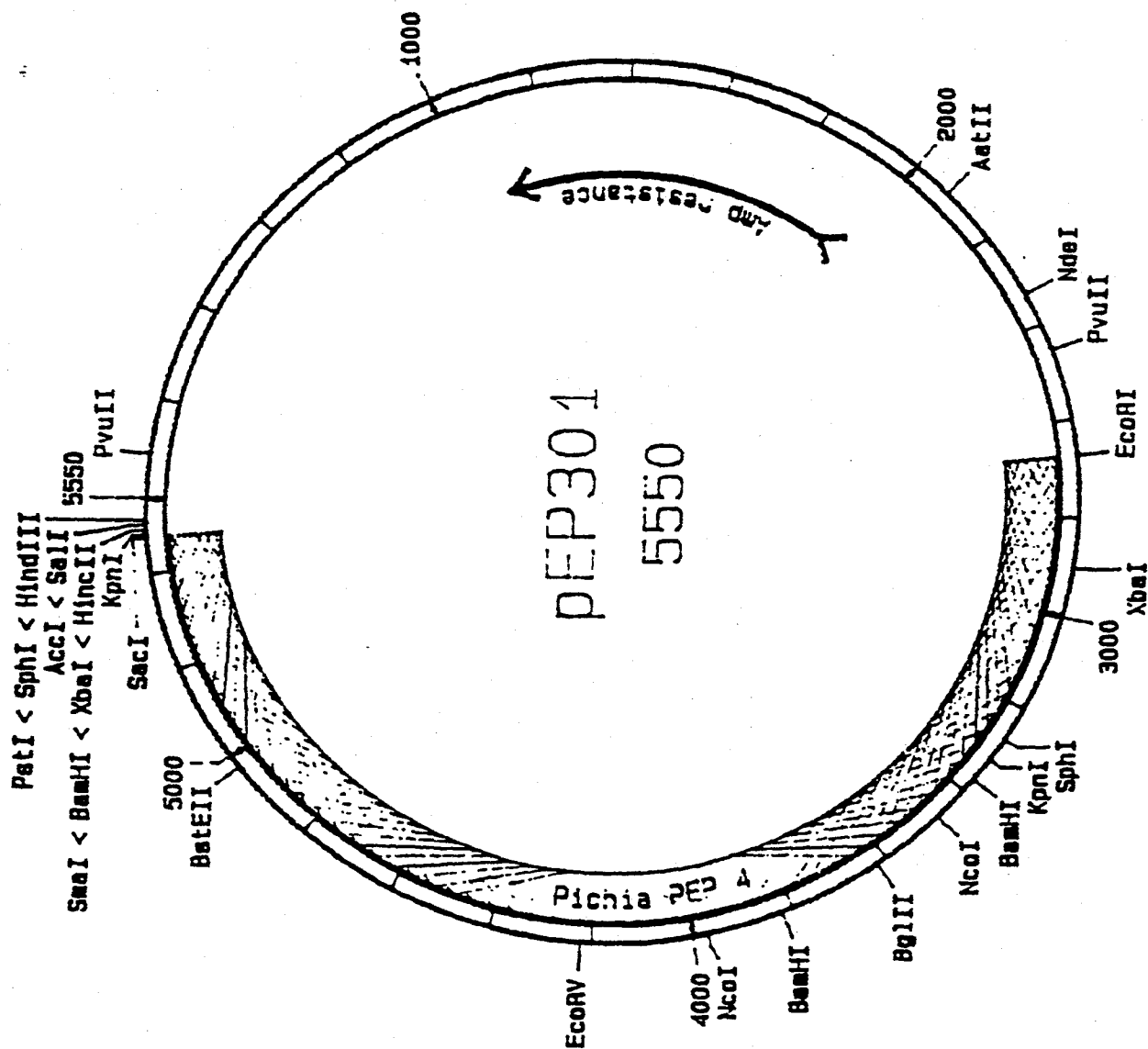


FIG
4

_ 7 / 2 3

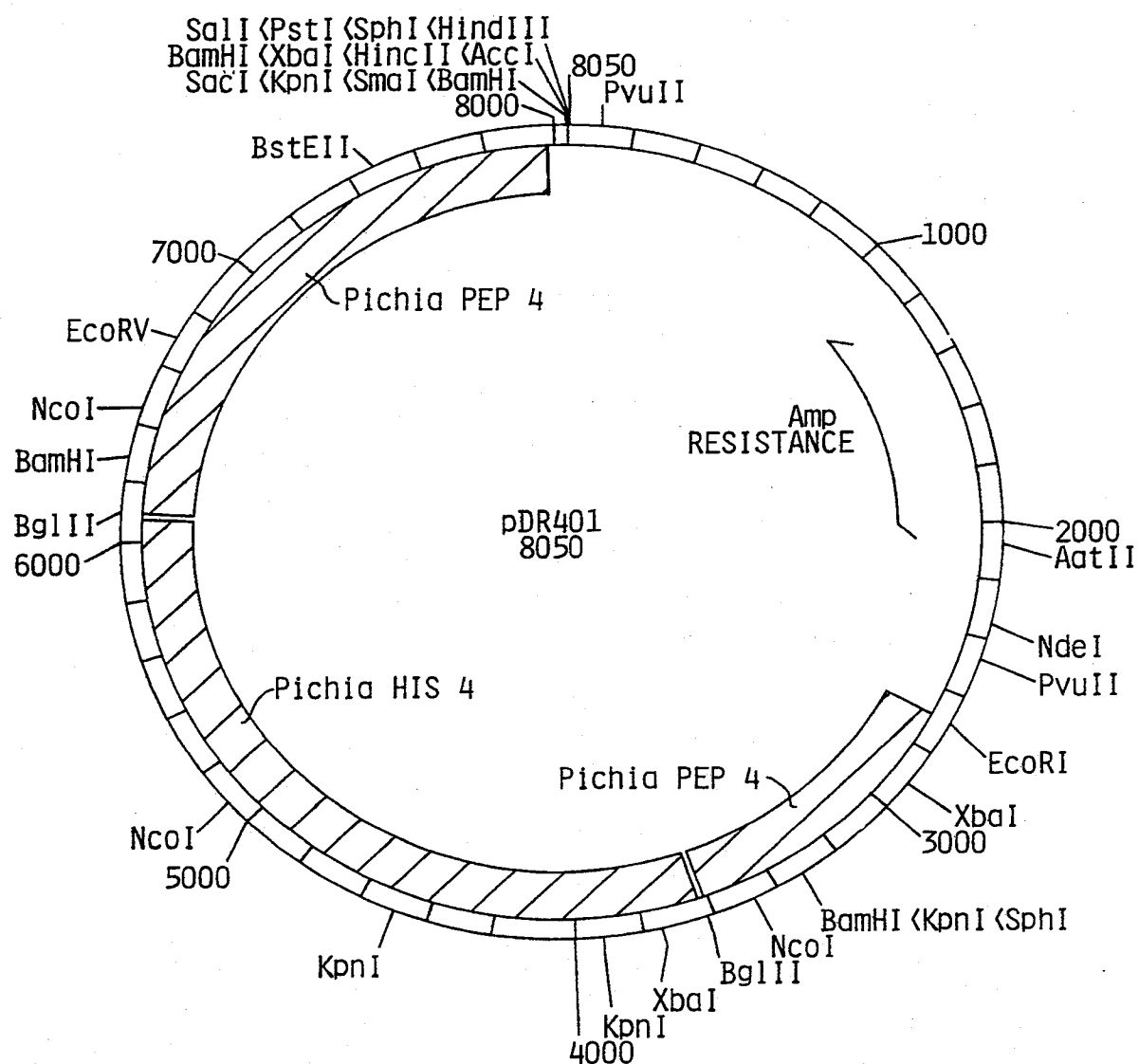


FIG. 5

SUBSTITUTE SHEET

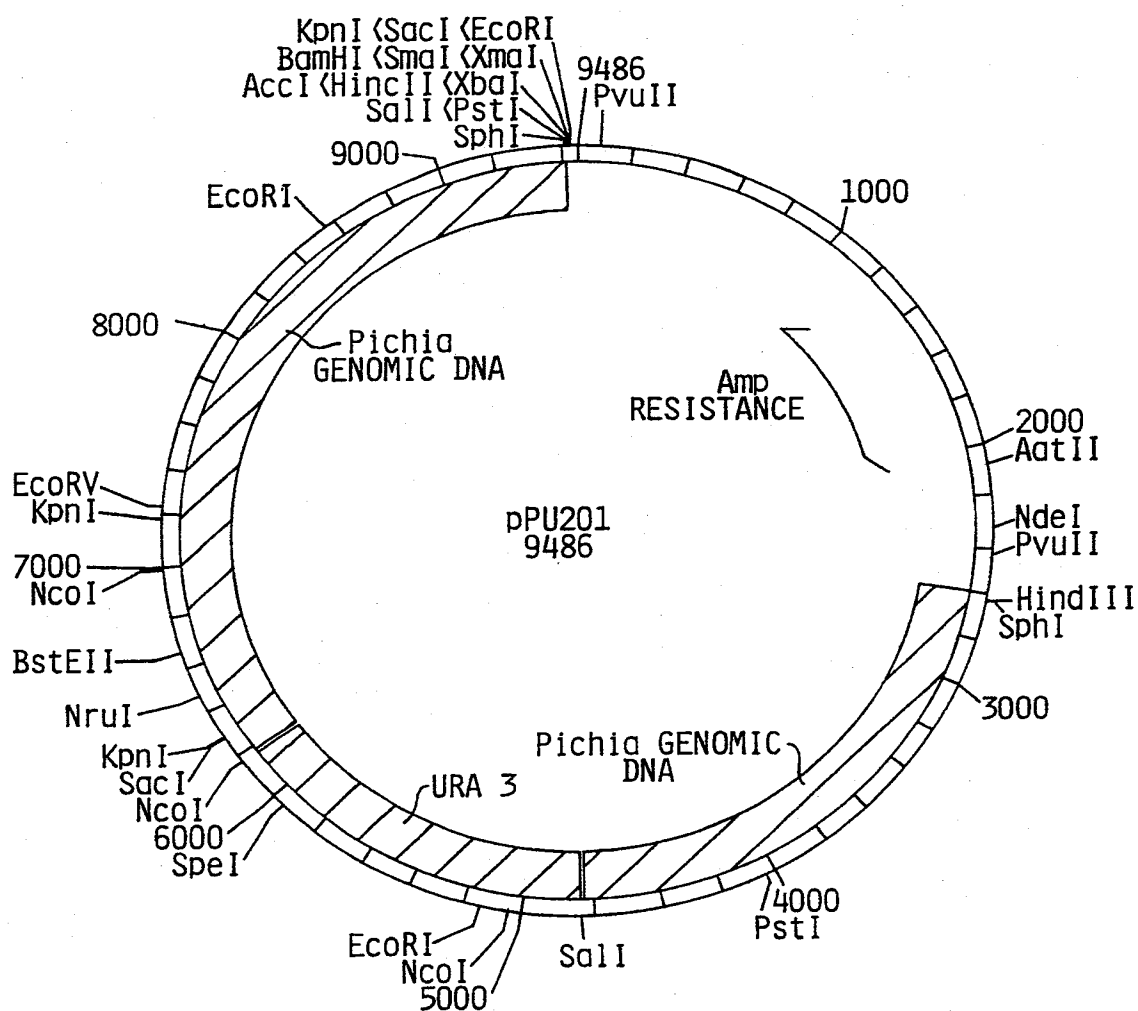
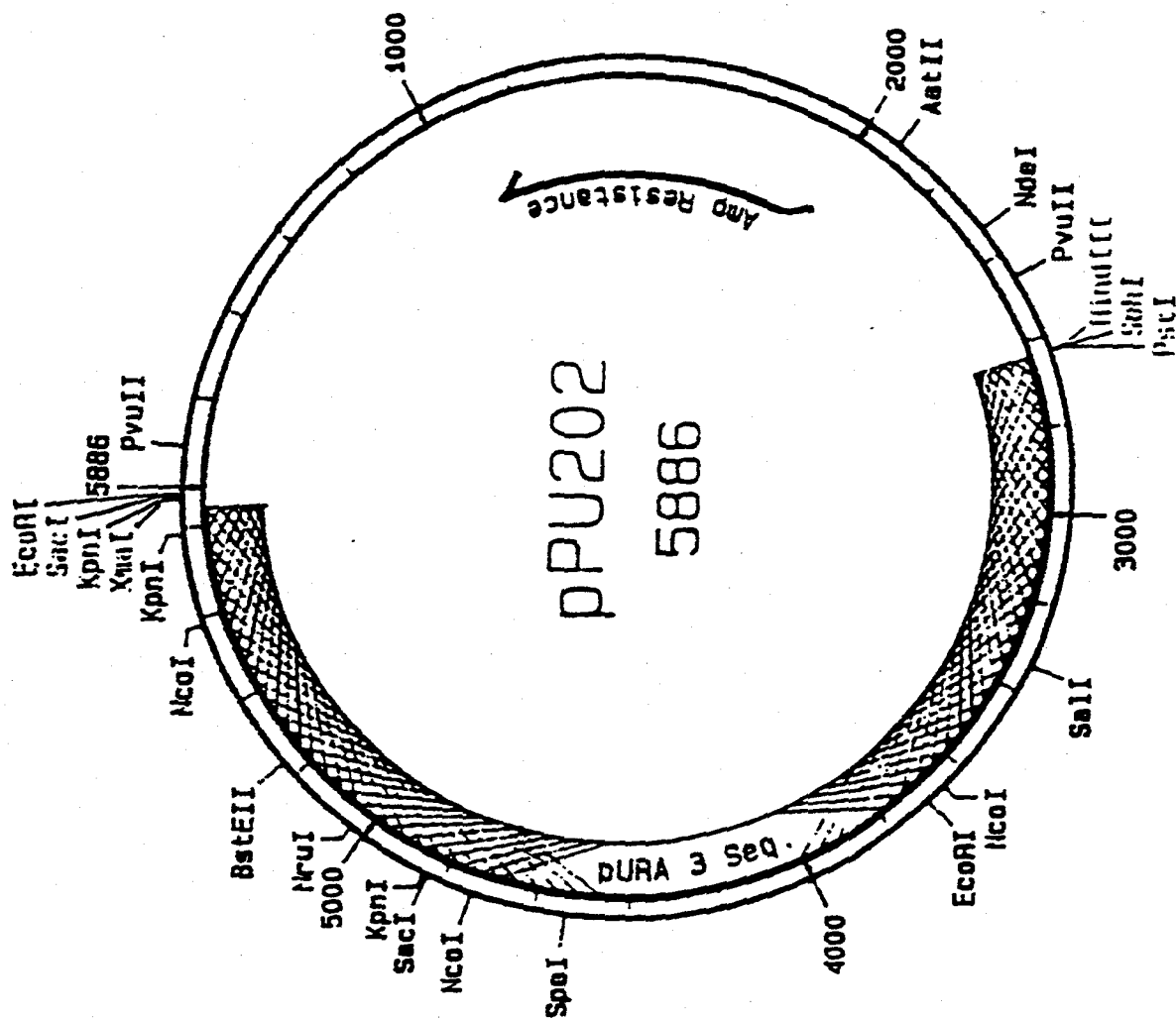


FIG. 6

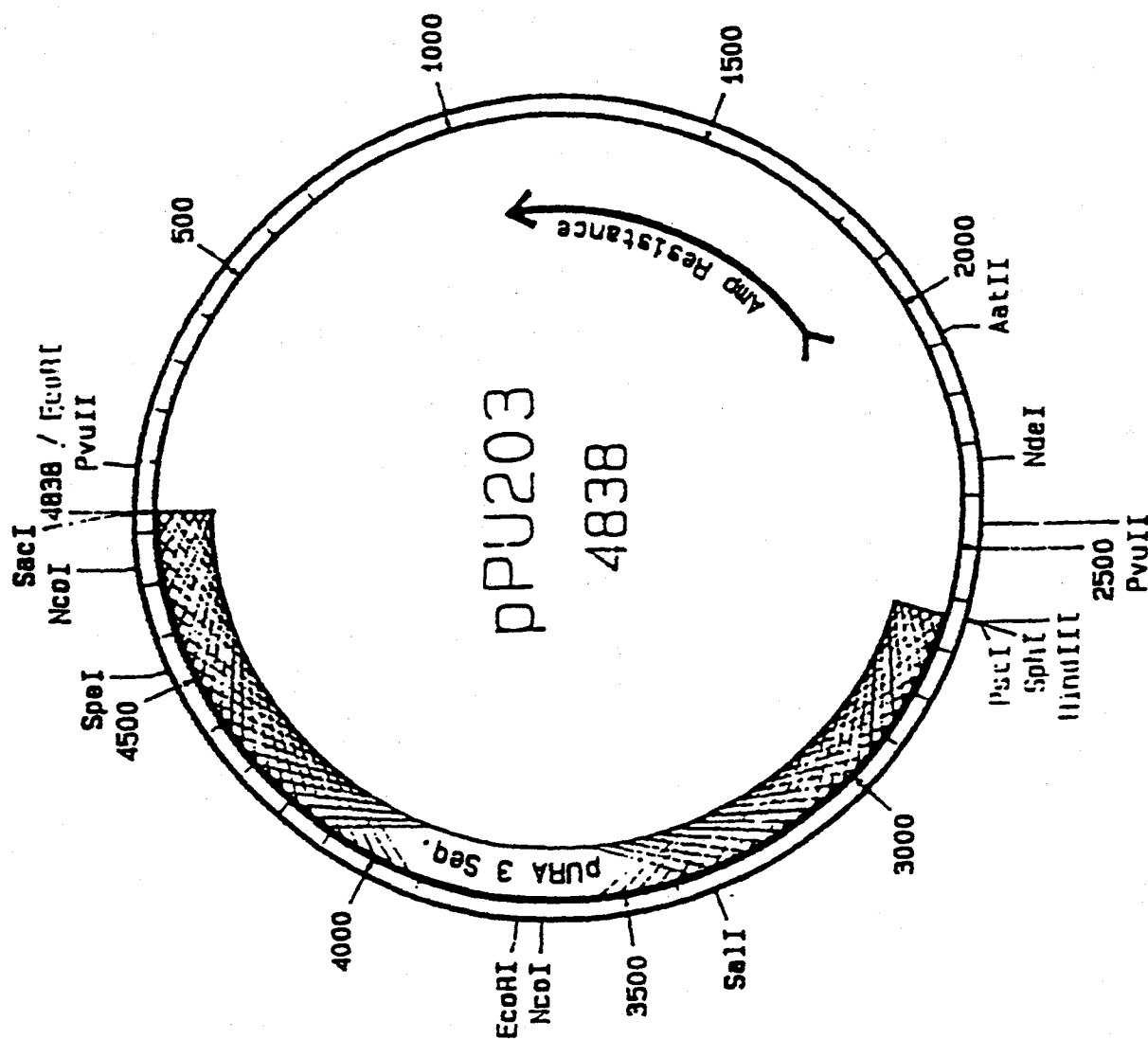
SUBSTITUTE SHEET

FIG 7



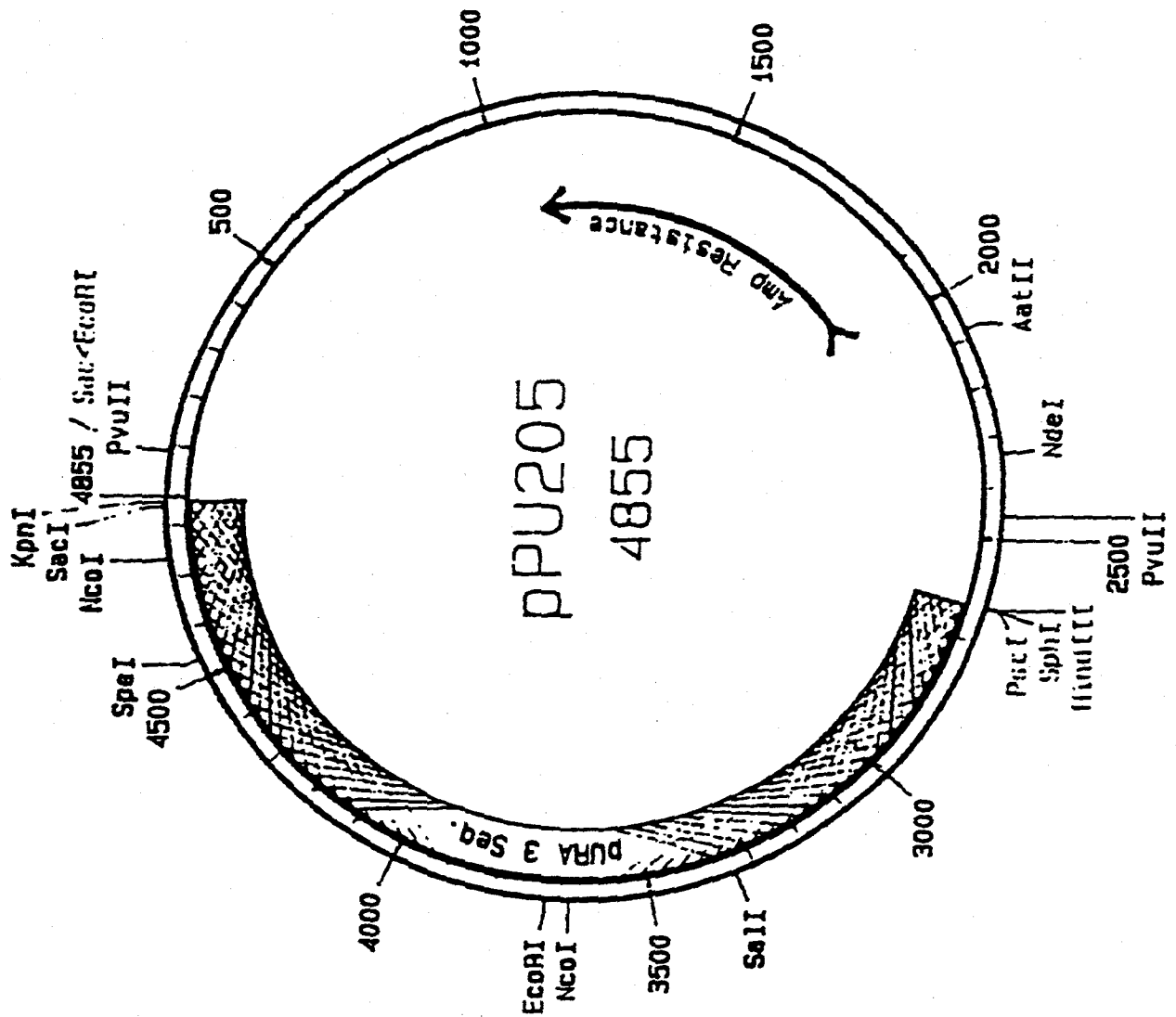
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Howard April 4, 1990 08:59

FIG
8

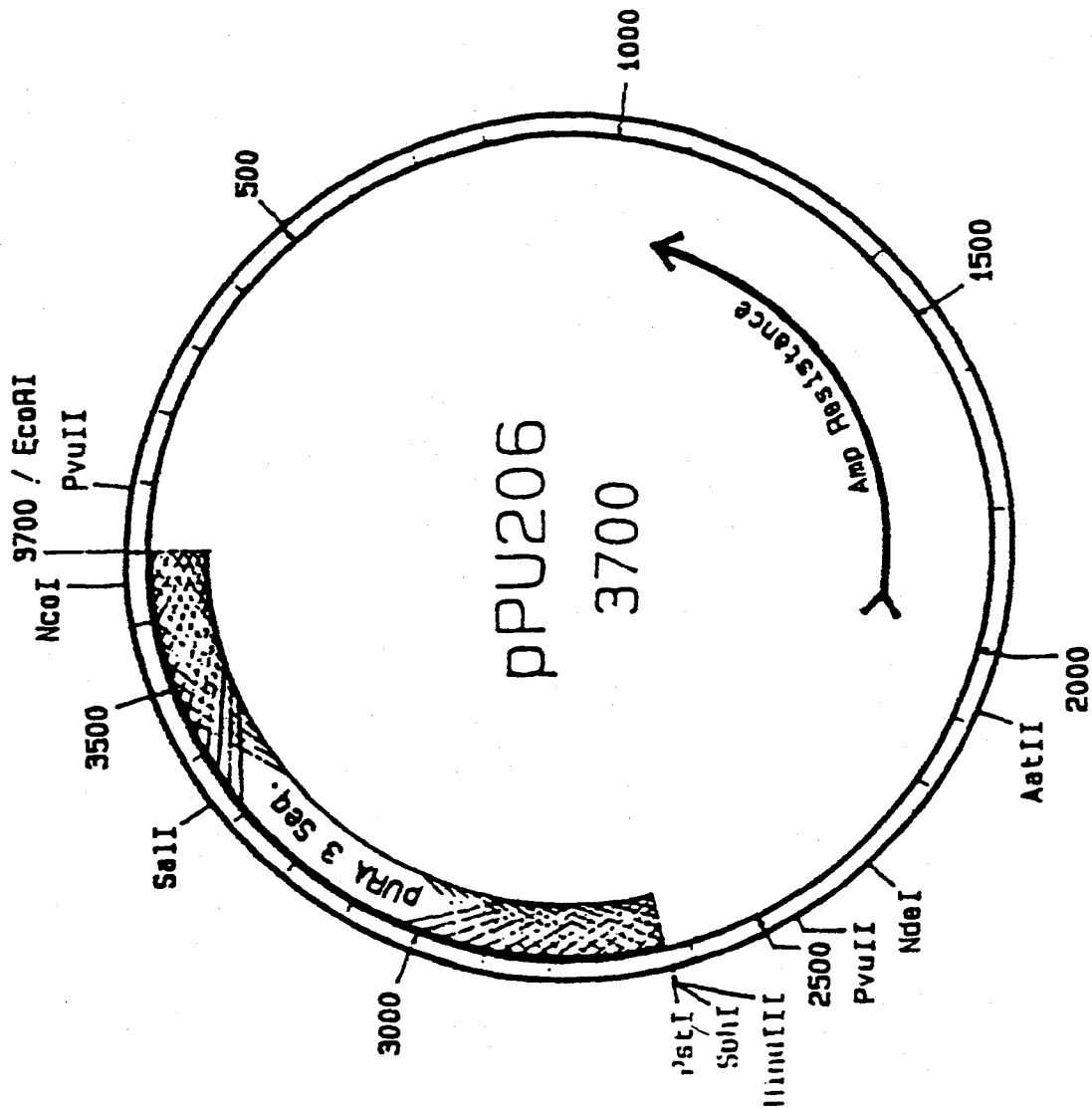
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Howard April 4, 1990 10:19

FIG
9

PLASMIDMAP of: pPU205.Seq check: 5464 from : to: 4855

Howard April 4, 1990 10:33

FIG
10

PLASMIDMAP of: pPU206. Seq check: 5464 from: 1 to: 3700

Howard April 4, 1990 10:39

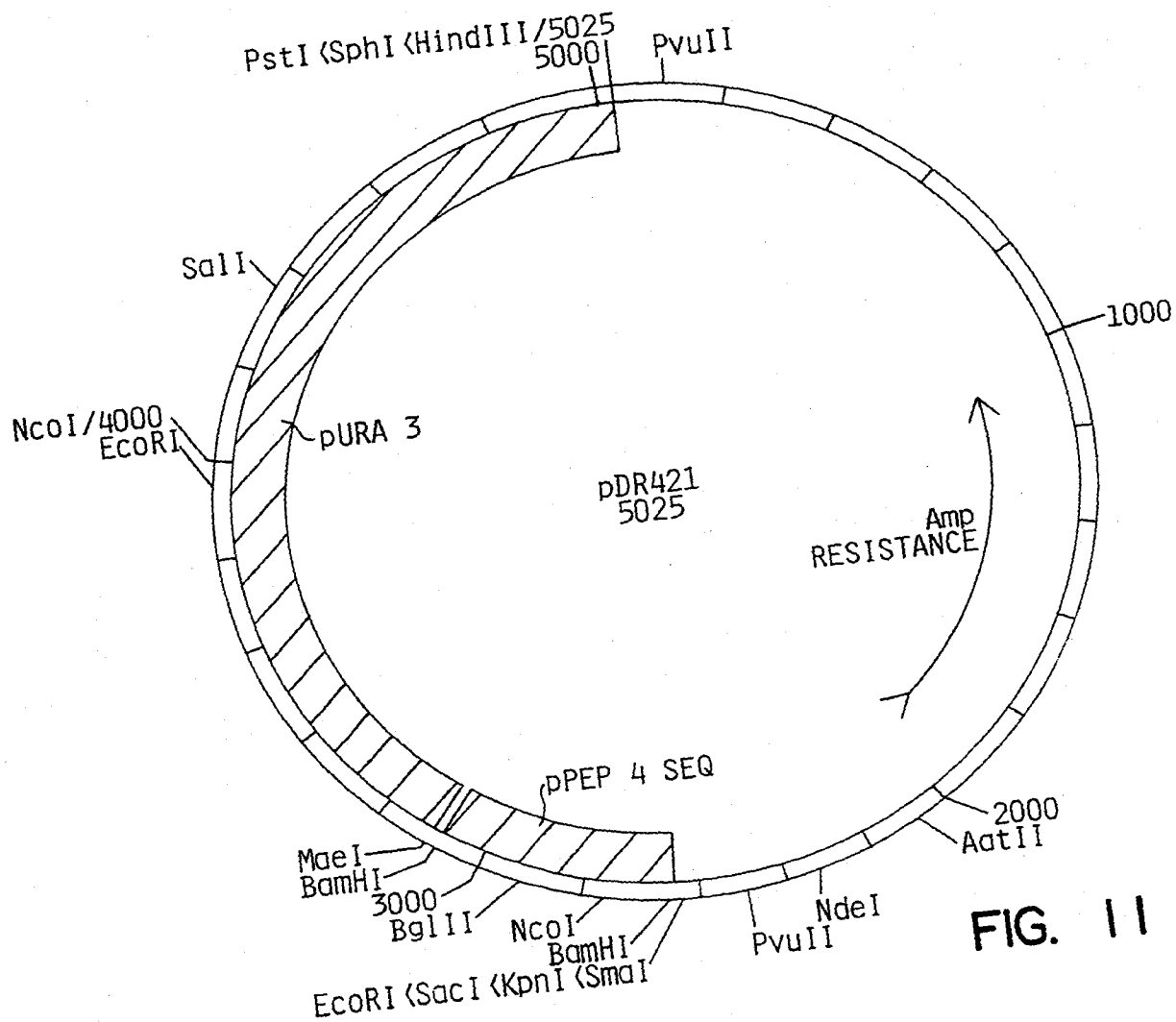
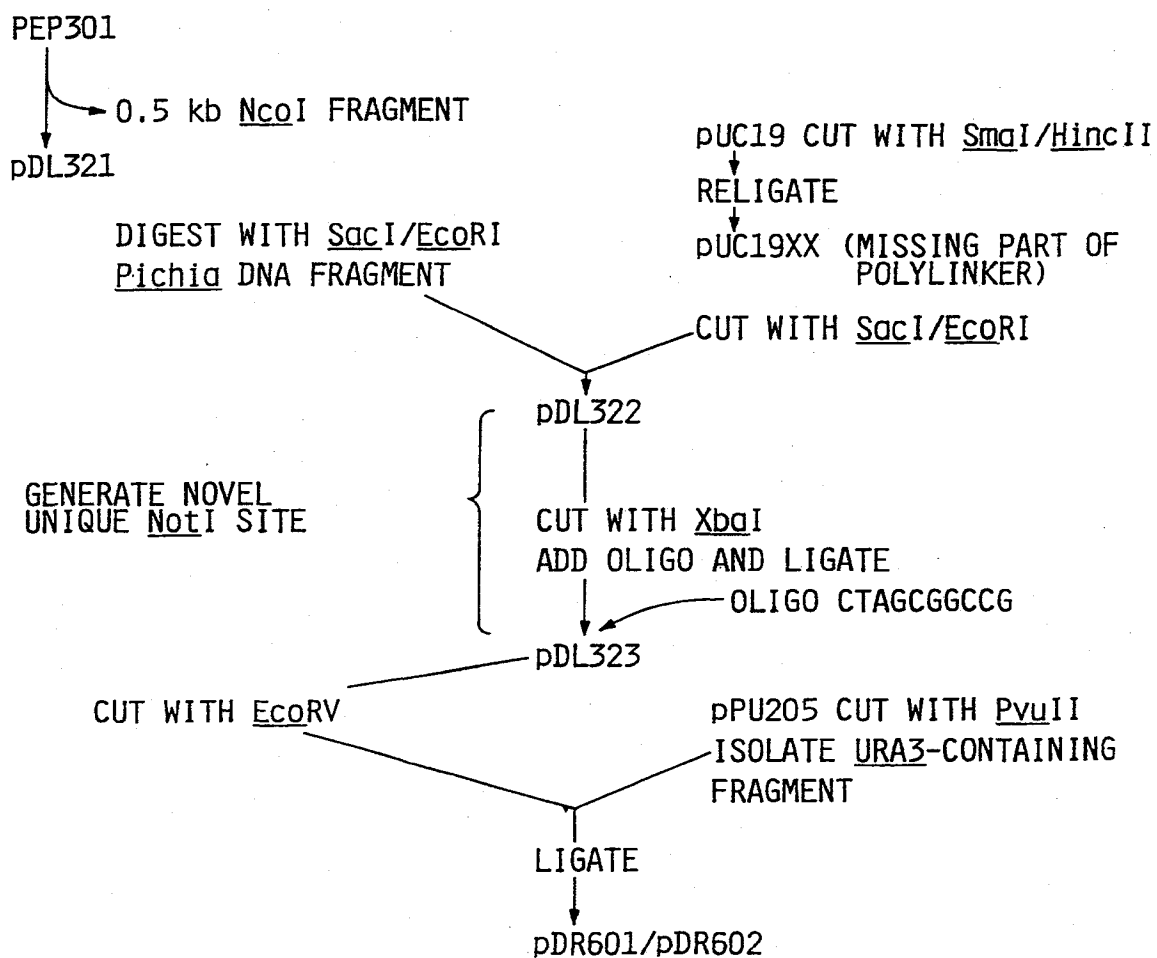


FIG. 11



SUBSTITUTE SHEET

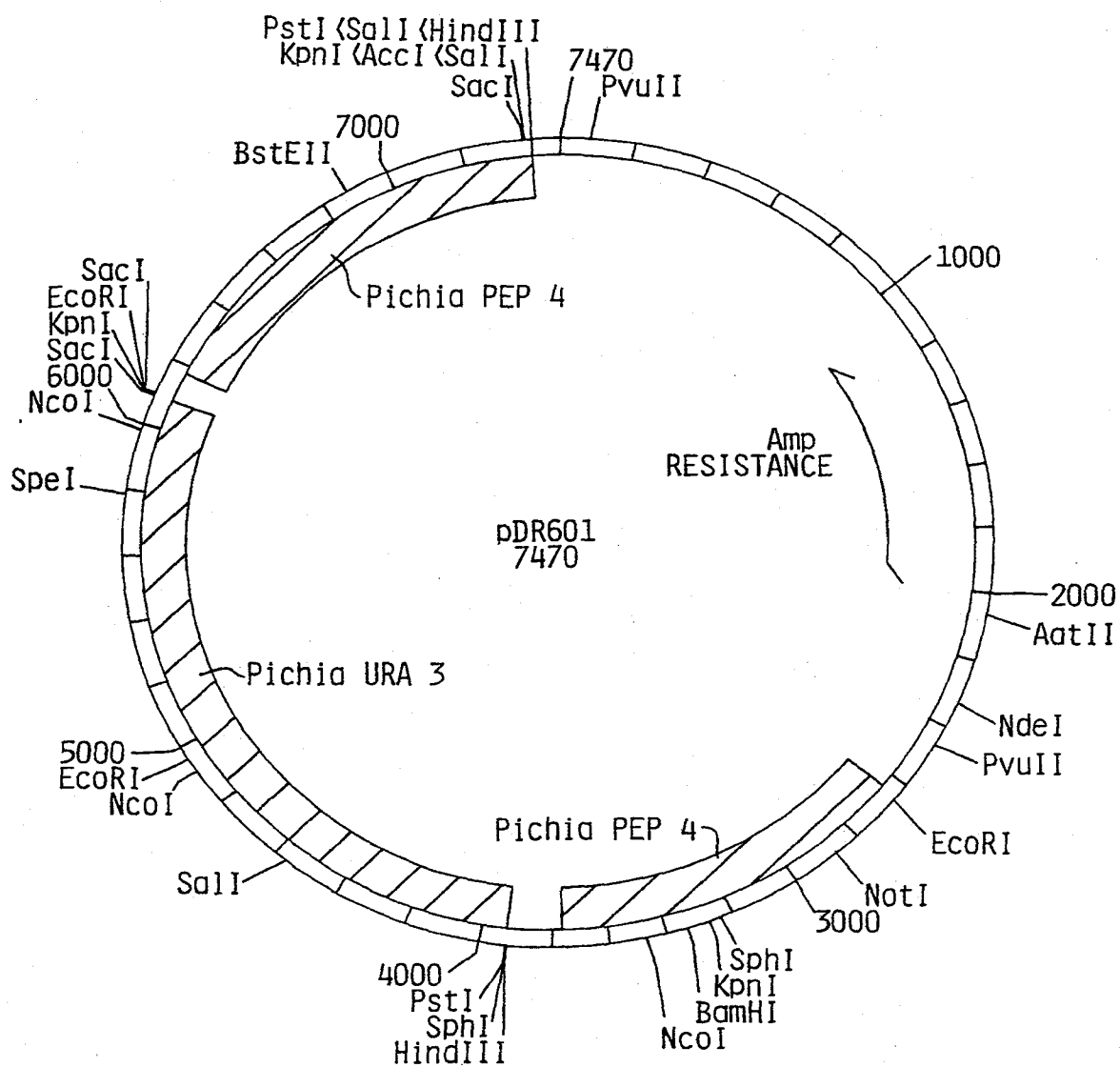


FIG. 14

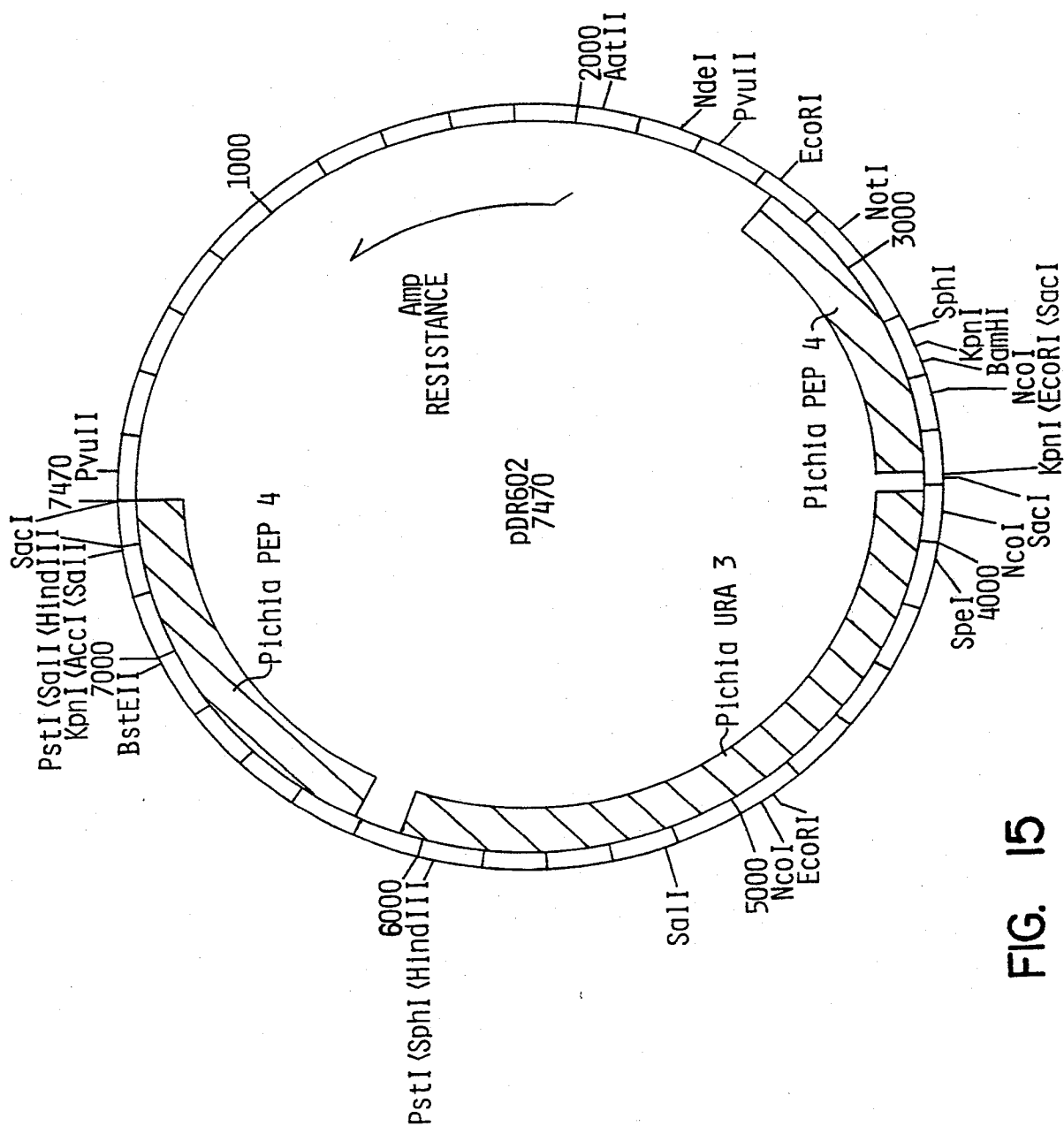


FIG. 15

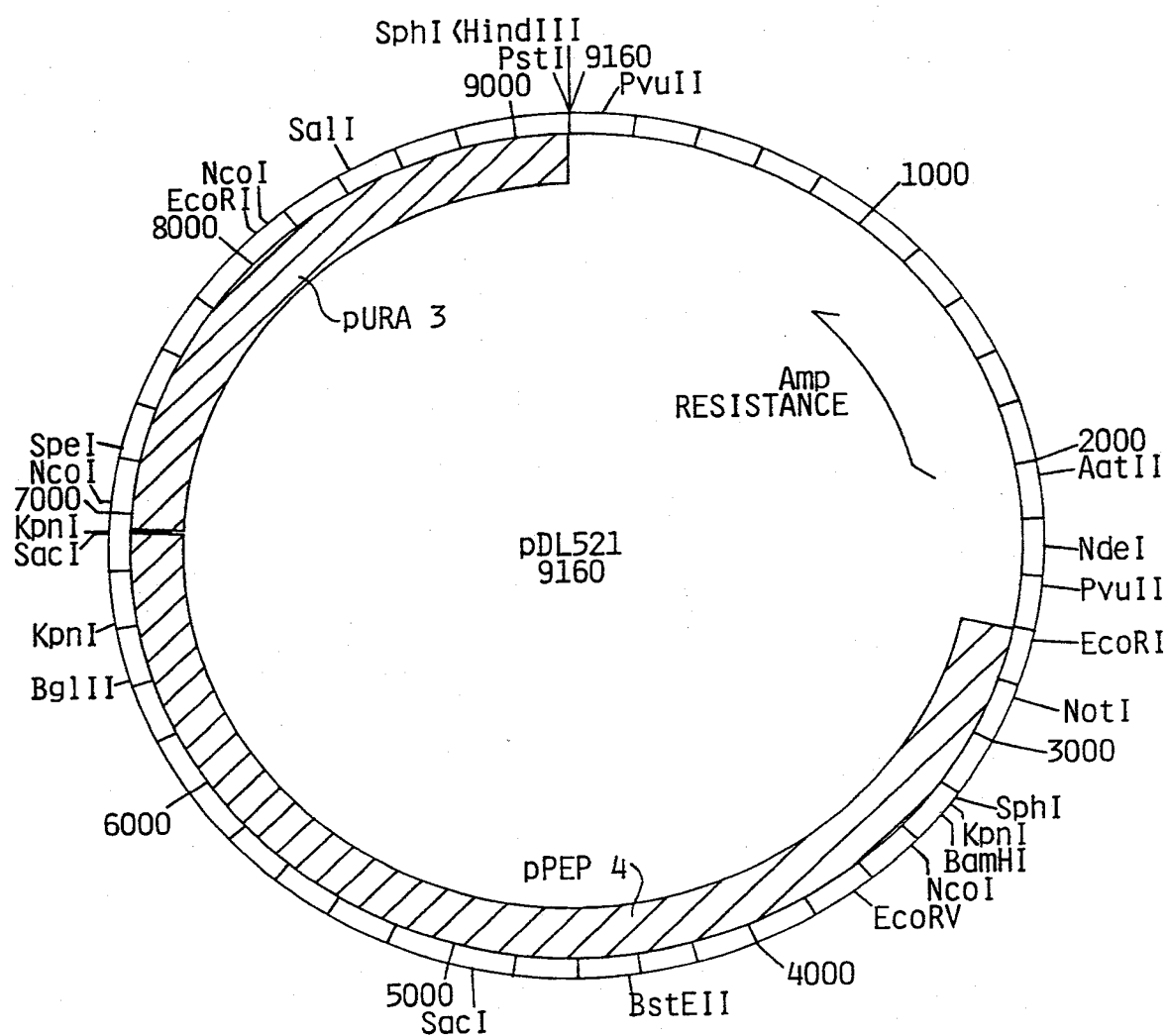
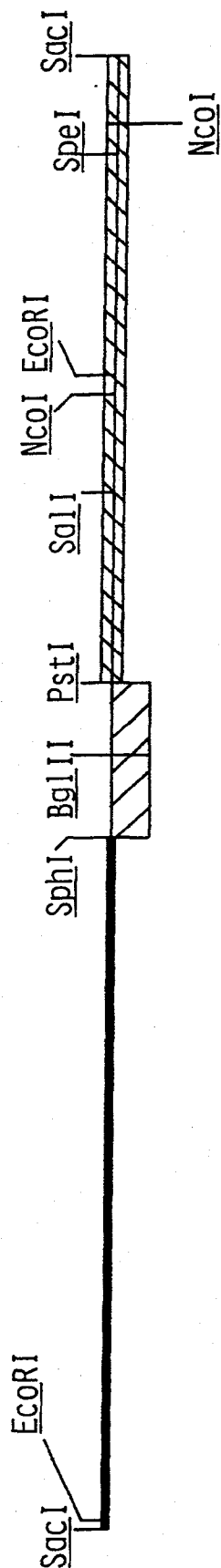


FIG. 16

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 PRB-1 GENE SEQUENCE
 URA3 GENE SEQUENCE
 pUC19

FIG. 18

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/81; C12N1/19;	C12N15/57; C07K13/00	C12N15/53; C12N15/60
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	EP,A,0 336 056 (M & D RESEARCH CO) 11 October 1989 see page 1, last paragraph - page 2; claim 2 ---	1
A	CHEMICAL ABSTRACTS, vol. 106, 1987, Columbus, Ohio, US; abstract no. 48594, G. NELSON AND T. YOUNG: 'Yeast extracellular proteolytic enzymes for chill-proofing beer' page 505 ; column L ; see abstract & J. INST. BREW. vol. 96, no. 6, 1986, pages 599 - 603; --- -/--	1
<p>¹⁰ Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11 AUGUST 1992	20. 08. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 390 676 (TRANSGENE S.A.) 3 October 1990 see the whole document ---	8,9,11, 12,14, 16,17, 19,22, 23,26, 28-30
A	BIOTECHNOLOGY vol. 7, no. 2, February 1989, NEW YORK US pages 160 - 164; M. DIGAN ET AL: 'Continuous production of a novel lysozyme via secretion from the yeast, Pichia pastoris' see the whole document ---	32
A	WO,A,9 009 449 (HENKEL RESEARCH CORPORATION) 23 August 1990 see abstract; claims ---	22,31, 32,34, 39-48
A	GENE. vol. 29, 1984, AMSTERDAM NL pages 113 - 124; M. ROSE: 'Structure and function of the yeast URA3 gene: expression in Escherichia coli' see the whole document ---	39
A	MOLECULAR AND CELLULAR BIOLOGY vol. 6, no. 7, July 1986, WASHINGTON, US pages 2490 - 2499; G. AMMERER ET AL: 'PEP4 gene of Saccharomyces cerevisiae encodes Proteinase A, a vacuolar enzyme required for processing of vacuolar precursors' see abstract; figure 2 ---	2,5,10, 18,20, 24,29, 31,36,47

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9202521
SA 59408

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 11/08/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0336056	11-10-89	JP-A- 1191683	01-08-89
		JP-A- 2049585	19-02-90
		US-A- 5053333	01-10-91
EP-A-0390676	03-10-90	FR-A- 2645175	05-10-90
		CA-A- 2013240	30-09-90
		JP-A- 3072868	28-03-91
WO-A-9009449	23-08-90	EP-A- 0457852	27-11-91

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国際事務局



(43) 国際公開日
2001年3月1日 (01.03.2001)

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WO 01/14522 A1

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(21) 国際出願番号: PCT/JP00/05474

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特願平11/233215 1999年8月19日 (19.08.1999) JP

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(81) 指定国 (国内): AU, CA, CN, JP, KR, US.

(84) 指定国 (広域): ヨーロッパ特許 (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

添付公開書類:
— 国際調査報告書

2文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: NOVEL YEAST VARIANTS AND PROCESS FOR PRODUCING GLYCOPROTEIN CONTAINING MAMMALIAN TYPE SUGAR CHAIN

(54) 発明の名称: 新規な酵母変異株および哺乳類型糖鎖を含有する糖タンパク質の製造法

(57) Abstract: Novel yeast variants capable of producing a glycoprotein wherein a sugar chain having the same sugar chain structure as a sugar chain produced by mammalian cells is attached to an asparagine residue of a protein; and a process for producing the sugar chain and the glycoprotein by a sugar chain engineering technique with the use of these variants. By using the auxotrophic triplex or tetraplex variant newly bred, a neutral sugar chain identical with the high-mannose type produced by mammalian cells (human cells, etc.) or a glycoprotein having the same neutral sugar chain can be efficiently produced at a high purity. By transferring a mammalian type sugar chain biosynthesis-associated gene into such a variant, it is also possible to efficiently produce a mammalian sugar chain of high-mannose type, hybrid type, complex type, etc. or a protein having a mammalian type sugar chain.

[続葉有]

WO 01/14522 A1



(57) 要約:

哺乳類細胞の生産する糖鎖と同一の糖鎖構造をもつ糖鎖をタンパク質のアスパラギン残基に付加した糖タンパク質を生産することのできる新規な酵母変異株、ならびに該変異株を用いて糖鎖工学的手法により糖鎖および糖タンパク質を製造する方法を提供する。

本発明により新規に育種した栄養要求性三重変異株、栄養要求性四重変異株によれば、ヒトなど哺乳類細胞の生産するハイマンノース型と同一の中性糖鎖、あるいは同一の中性糖鎖を有する糖タンパク質を多量かつ純度よく生産することができる。また、当該変異株に哺乳類型糖鎖の生合成系遺伝子を導入することにより、ハイマンノース型、ハイブリッド型、複合型等の哺乳類型糖鎖、あるいは哺乳類型糖鎖を有するタンパク質を効率的に生産することができる。

明 細 書

新規な酵母変異株および哺乳類型糖鎖を含有する糖タンパク質の製造法

技術分野

本発明は、哺乳類細胞の生産する糖鎖と同一の糖鎖構造を有する糖鎖をタンパク質のアスパラギン残基に付加した糖タンパク質生産能を有する新規な酵母変異株、ならびに該変異株を用いて糖鎖工学的手法により糖鎖および糖タンパク質を製造する方法に関する。

背景技術

自然界に存在するタンパク質には、アミノ酸のみからなる単純タンパク質と、糖鎖や脂質、リン酸などが結合した複合タンパク質の2種類があり、サイトカイン類に関してはそのほとんどが糖タンパク質であることが知られている。このうち、エリスロポエチン（EPO）や組織プラスミノーゲン活性化因子（TPA）などについては、その糖鎖を除くと本来の生物活性を示さなくなることが明らかにされてきた（木幡陽、蛋白質核酸酵素、36, 775-788 (1991)）。糖鎖が生物活性の発現に重要な役割を担っていることが予想されるが、糖鎖の構造と生物活性との相関が必ずしも明確でないため、タンパク質部分に付加する糖鎖の構造（糖の種類、結合位置、鎖長など）を自由自在に改変制御できる技術の開発が必要となる。

糖タンパク質の糖鎖には、大別して、Asn結合型、ムチン型、O-GlcNAc型、GPIアンカー型、プロテオグリカン型などがあり（竹内誠、グリコバイオロジーシリーズ5、グリコテクノロジー、木幡陽・箱守仙一郎・永井克孝編、講談社サイエンティフィック、191-208 (1994)）、それぞれ固有の生合成経路を持ち、個別の生理機能を担っている。このうち、Asn結合型糖鎖の生合成経路については多くの知見があり、詳しく解析されている。

Asn結合型糖鎖の生合成は、N-アセチルグルコサミン、マンノース、およびグルコースからなる前駆体が脂質キャリアー中間体の上に合成され、まず小胞体

(ER) で糖タンパク質の特定の配列 (Asn-X-SerまたはThr) に転移される。次にプロセッシング (グルコース残基と特定のマンノース残基の切断) を受け、マンノース 8 残基と N-アセチルグルコサミン 2 残基からなる M8 ハイマンノース型糖鎖 ($\text{Man}_8\text{GlcNAc}_2$) が合成される。このハイマンノース型糖鎖を含有するタンパク質はゴルジ体に輸送されて、種々の修飾を受けるが、このゴルジ体での修飾は酵母と哺乳類で大きく異なっている (Gemmill, T.R., Trimble, R.B., *Biochim. Biophys. Acta.*, 1426, 227 (1999))。

哺乳類細胞では、多くの場合、ハイマンノース型糖鎖に α -マンノシダーゼ I が作用してマンノース数残基を切断する。この過程で生成する糖鎖 ($\text{Man}_5\text{GlcNAc}_2$) は、ハイマンノース型と呼ばれる糖鎖である。マンノースが 3 残基切断された M5 ハイマンノース型糖鎖 ($\text{Man}_5\text{GlcNAc}_2$) に N-アセチルグルコサミニルトランスフェラーゼ (GnT) I が作用し、N-アセチルグルコサミンを 1 残基転移し、 $\text{GlcNAcMan}_5\text{GlcNAc}_2$ からなる糖鎖が生成する。このようにしてできた糖鎖は混成 (ハイブリッド) 型と呼ばれる。更に、 α -マンノシダーゼ II、GnT-II が作用すると、 $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ という複合 (コンプレックス) 型と呼ばれる糖鎖構造となり、これ以降、十数種にもおよぶ糖転移酵素群が作用して、N-アセチルグルコサミン、ガラクトース、シアル酸等を付加し、多様な哺乳類型糖鎖を形成する (図 1)。哺乳類ではハイマンノース型、混成型、複合型いずれの糖鎖も見られるが、タンパク質によってその結合する糖鎖が異なっていたり、また一つのタンパク質内でも型の異なる糖鎖が結合していたりする。これらの糖鎖は、その型や結合している糖鎖の種類によって糖タンパク質の生合成、細胞内ソーティング、抗原性の隠蔽、生体内安定性、臓器ターゲティング特性などの優れた機能を示す (遠藤玉夫、糖鎖工学、産業調査会、64-72 (1992))。

遺伝子組換え体動物細胞を宿主として生産された史上初の糖タンパク質型医薬品となったエリスロポエチンについては、その糖鎖の重要性が指摘されている。エリスロポエチンの糖鎖は受容体との結合には阻害的に働くが、活性構造の保持、および体内動態の改善に決定的な寄与があり、全体として薬理活性の発現に必要不可欠であることが示された (Takeuchi and Kobata, *Glycobiology*, 1, 337-346 (1991))。更に、糖鎖の構造、種類、分岐数 ($\text{Man}_3\text{GlcNAc}_2$ に結合する GlcNAc

によって形成される枝分かれの数) とエリスロポエチンの薬理効果との間に強い相関性が見いだされた (Takeuchi et al., Proc. Natl. Acad. Sci. USA, 86, 7819-7822 (1989))。分岐構造の発達していないエリスロポエチンでは腎でのクリアランスが早まり、結果として体内滞留時間が短くなることがこの現象の主な原因であると報告されている (Misaizu et al., Blood, 86, 4097-4104 (1995))。これに似た例はフェツインなどの血清糖タンパク質でも見られ、糖鎖の末端のシアル酸を除去することでガラクトースが露出すると、肝細胞表面のレクチンによって認識され、血中から速やかに消失してしまうことが見いだされている (Ashwell and Harford, Annu. Rev. Biochem., 51, 531-554 (1982) ; Morell et al., J. Biol. Chem., 243, 155-159 (1968))。

また、ヒトリソソームに局在する酵素群の多くは、生合成されゴルジ体に輸送されると、そのハイマンノース型糖鎖の非還元末端のマンノース残基の6位にリン酸基が付加され、これがリソソーム酵素特異的な認識マーカーとなる。そしてその高親和性受容体であるマンノース-6-リン酸受容体 (MPR) との結合を介して、他のタンパク質から選別され、プレリソソームへ運ばれ、酸性条件下でMPRから解離した後、更にリソソームへと輸送される (von Figura and Hasilik, Annu. Rev. Biochem., 54, 167-193 (1984))。このリソソーム酵素特異的なリン酸基の付加反応は、2種の酵素反応により行なわれており、これらの遺伝子に遺伝的欠陥を有する場合、リソソームへのターゲティング機構に異常が生じ、リソソーム病と総称される重篤な病態を生じることが知られている (Leroy and DeMars, Science, 157, 804-806 (1967))。したがって、哺乳類型糖鎖と一口に言っても、その構造が機能に大きく関わっていると言える。

一方、酵母では、M8ハイマンノース型糖鎖にマンノースが数残基から100残基以上付加した、マンナン型糖鎖 (外糖鎖) を生成する。Saccharomyces属酵母における外糖鎖の生合成は図2、3で示したような経路で進行すると考えられている (Ballou et al., Proc. Natl. Acad. Sci. USA, 87, 3368-3372 (1990))。すなわち、M8ハイマンノース型糖鎖にまず α -1,6結合でマンノースが付加する延長開始反応が起こる (図2, 反応I, B)。この反応を行なう酵素はOCH1遺伝子にコードされるタンパク質であることが明らかになっている (Nakayama et al.,

EMBO J., 11, 2511-2519 (1992))。更に α -1,6結合でマンノースを逐次延長する反応(図2, II)が起こることにより、外糖鎖の骨格となるポリ α -1,6結合マンノース結合が形成される(図2, E)。この α -1,6結合のマンノースには、 α -1,2結合したマンノースの分岐が存在し(図2, 3 : C, F, H)、この枝分かれした α -1,2結合のマンノースの先には、更に α -1,3結合したマンノースが付加することがある(図2, 3 : D, G, H, I)。この α -1,3結合のマンノースの付加は、MNN1遺伝子産物によるものである(Nakanishi-Shindo et al., J. Biol. Chem., 268, 26338-26345 (1993))。またハイマンノース型糖鎖部分および外糖鎖部分にマンノース-1-リン酸が付加した酸性糖鎖も生成することがわかっている(図2, * ; 前記式(I)中の*に対応するリン酸化可能部位)。この反応はMNN6遺伝子がコードする遺伝子によることがわかり(Wang et al., J. Biol. Chem., 272, 18117-18124 (1997))、更にこの転位反応を正に制御するタンパク質をコードする遺伝子(MNN4)も明らかとなった(Odani et al., Glycobiology, 6, 805-810 (1996) ; Odani et al., FEBS letters, 420, 186-190 (1997))。

多くの場合に、外糖鎖は不均質なタンパク質産物を生成し、タンパク質の精製を困難にしたり、比活性を低下させたりする(Bekkers et al., Biochim. Biophys. Acta, 1089, 345-351 (1991))。更に糖鎖の構造が大きく異なるため、酵母で生産された糖タンパク質は、哺乳類由来のものと同一の生物活性が検出されなかったり、哺乳類動物などに対して強い免疫原性を有する。このように、哺乳類由来の有用糖タンパク質を生産させる際の宿主としては、酵母は不適当とされている。哺乳類由来のものと同等の生物活性を持った糖タンパク質、すなわち哺乳類型の糖鎖を含有する糖タンパク質を生産できる酵母の開発が、学会や産業界から望まれている。

従って、酵母を用いて哺乳類型糖鎖を生産するためには、まず、前記のような酵母特有の糖タンパク質糖鎖の修飾であるマンノースを多数付加するような反応がおこらず、外糖鎖が付加しなくなり、糖鎖合成がM8ハイマンノース型糖鎖で停止するような糖鎖生合成系を有する変異株を単離することが重要となる。次に、この哺乳類型糖鎖の前駆体であるM8ハイマンノース型糖鎖に、哺乳類型糖鎖の生合成系遺伝子を上記酵母変異株に導入することにより達成されるはずである。

そこで、以前より外糖鎖が欠失した糖タンパク質を得るために、酵母外糖鎖生成系酵素群の欠損株の使用が検討されてきている。欠損株を得るためには、薬剤や紫外線照射、自然変異により遺伝子突然変異株を取得する場合と、人為的に標的遺伝子を破壊する方法がある。

前者についてはこれまで様々な報告がある。例えば、mnn2変異株は外糖鎖の α -1,6骨格から α -1,2結合を生じる枝分かれのステップに欠損があり、mnn1変異株は分岐先端に α -1,3結合のマンノースを生成するステップに欠損がある。しかし、これらの変異株は外糖鎖の骨格である α -1,6マンノース結合には欠損がないため、いずれも鎖長の長い外糖鎖を生成する。またmnn7, 8, 9, 10変異株などは α -1,6マンノース結合を4-15分子程度しか持たない変異株として単離されているが、これらの変異株も外糖鎖が短くなるだけであり、ハイマンノース型糖鎖で糖鎖伸長が停止するものではない (Ballou et al., J. Biol. Chem., 255, 5986-5991 (1980); Ballou et al., J. Biol. Chem., 264, 11857-11864 (1989))。外糖鎖の付加欠損は、小胞体からゴルジ体へのタンパク質輸送が温度感受性となったsec18などの分泌変異株などでも観察される。しかし、sec変異株では、タンパク質の分泌そのものが高温で阻害されてしまうため、糖タンパク質の分泌生産という目的にはそぐわない。

よって、これらの変異株は目的のハイマンノース型糖鎖を完全には生合成できないため哺乳類糖鎖を生成するための宿主酵母としては不適であると考えられる。

一方、酵母における小胞体 (ER) での糖鎖生合成経路は、生合成が種々の段階で欠損した変異株を単離し、これを生化学的に解析することによって明らかにされてきた。alg (asparagine-linked glycosylation) 変異株は、糖鎖への $[^3\text{H}]$ マンノースの取り込みが糖外鎖を持つ野生型細胞より少なくなったため、放射線による損傷・死滅をまぬがれた変異株を濃縮するという巧妙な選別法により単離された。その中でalg3変異は非許容温度下でDol-pp-GlcNAc₂-Man₅ (Dol-ppはドリコールピロリン酸) を蓄積する (Tanner, W. et al., Biochim. Biophys. Acta., 906, 8199 (1987))。また地神らは Δ och1mnn1alg3三重変異株を用いて解析を行っている (地神ら、蛋白質 核酸 酵素、Vol. 39, No. 4, p.657 (1994))。マンナン蛋白質糖鎖をPA (2アミノピリジイン) で蛍光ラベル後、分析すると、

主成分は $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ および $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ と一致する2つのピークを示した。このうち、前者は α -1,2-マンノシダーゼ消化やFAB-MSの結果などから、ERコア糖鎖と同一であることが判明した。一方、後者は α -1,2マンノシダーゼ消化によりManが2分子除去されて $\text{Man}_2\text{GlcNAc}_2\text{-PA}$ を生産し、 α -1,6結合したManを特異的に切断する処理（部分アセトリシス）により、Manが1分子だけ除去された。これらの結果から、この三重変異株の生産する $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ の糖鎖は前記式（II）に示した不完全なコア型糖鎖構造であることが判明した。なお、この三重変異株で $\text{Man}_5\text{GlcNAc}_2$ だけでなく $\text{Man}_8\text{GlcNAc}_2$ も生成する理由としては、ドリコールピロリン酸上で $\text{Man}_5\text{GlcNAc}_2\text{-pp-Dol}$ を蓄積する alg3 変異の性質がリーキー（leaky）なためである。

一方、後者については近年の遺伝子工学的手法の発達により、標的遺伝子を複数個破壊した欠損株を構築できるようになった。

酵母の中には栄養要求性変異を持つものが知られており、栄養要求性変異としては、 leu2 変異、 trp1 変異、 ura3 変異、 ade2 変異、 his3 変異などがある（大嶋泰治編著、生物化学実験法39、酵母分子遺伝学実験法、119-144（1996））。変異のない元の遺伝子を導入すれば、これらの栄養要求性を解除することができ、培地中に必要成分を加えなくても生育が可能となる。この原理を基に酵母の遺伝子破壊を行うことができる（図4）。この方法では、まず、試験管内での操作により、プラスミド上の標的遺伝子DNAを分断あるいは部分欠失させ、そこに適当な選択マーカー遺伝子DNAを挿入して標的遺伝子の上流部と下流部の間に選択マーカーがサンドイッチされた構造体を作製する。次に、この構造を持つ線状DNAを酵母細胞に導入することにより、導入断片の両端と染色体上の標的遺伝子との相同部分の間に2回の組み換えを起こし、選択マーカーを挟み込んだDNA構成体で置換するものである（Rothstein, *Methods Enzymol.*, 101, 202-211（1983））。この方法では、1つの遺伝子を破壊するのに1つの選択マーカーが必要となる。

外糖鎖を欠損した酵母株の分子育種は既に特開平6-277086や特開平9-266792に記載されている。しかし、特開平6-277086に記載された二重変異株（ Δoch1 Δmnn1 ）で生産された糖タンパク質糖鎖には、リン酸残基を持つ酸性糖鎖が含

まれていることがわかった。この酸性糖鎖はヒトなど哺乳類由来の糖鎖には存在しない構造であり、哺乳類の体内で異物と認識されて、抗原性を示すと思われる (Ballou, Methods Enzymol., 185, 440-470 (1990))。そこで、更にマンノース-1-リン酸転移を正に制御する遺伝子 (MNN4) およびO-結合型糖鎖の延長反応を行なうマンノース転移酵素遺伝子 (KRE2) の機能を破壊した四重変異株 (特開平9-266792に記載) が構築された。これに記載された酵母株の生産する糖タンパク質の糖鎖は、目的のM8ハイマンノース型糖鎖を有していることが明らかとなった。更にこの破壊酵母に *Aspergillus saitoi* 由来の α -1,2-mannosidase 遺伝子を導入した株は、マンノースが1〜数残基切断されたハイマンノース型糖鎖 ($\text{Man}_5\text{GlcNAc}_2$) を有することが明らかとなっている (Chiba et al., J. Biol. Chem., 273, 26298-26304 (1998))。

また、新聞らは更に *alg3* 変異を導入した別種の四重変異株を作成している (Shimma Y. et al., Mol. Gen. Genet., 256, 469-480 (1997); Wang et al., J. Biol. Chem. 272, 18117-18124 (1997); 新聞陽一、地神芳文、第32回酵母遺伝学フォーラム要旨集、p.64 (1999) ; Shimma Y. et al., Abstracts of XIX International Conference On Yeast Genetics and Molecular Biology, p. 443 (1999))。

ところが、これらの酵母株の生産する糖タンパク質の糖鎖は、哺乳類にも存在し抗原性を有さないが、エリスロポエチンの例からも明らかなように、ハイマンノース型糖鎖を含有する糖タンパク質は、その糖鎖構造から哺乳類細胞から生産された糖タンパク質と同等の活性を示さないことが予想される。また、この四重変異株は標的遺伝子の破壊により、劣性の遺伝子変異である宿主の4個の選択マーカー (*leu2*, *ura3*, *lys2*, *trp1*) の以後の使用が不可能となる。更に残った栄養要求性マーカーのうち1つについては、突然変異ではなく人為的に破壊されているため、染色体上のこれらのマーカー遺伝子座との相同性を利用した酵母染色体上への組み込みは行なえない。よって、これらの酵母株に対し、哺乳類型の糖鎖を生産するのに必要な糖鎖加水分解酵素遺伝子群、糖転移酵素遺伝子群、糖ヌクレオチド輸送体遺伝子群に属する遺伝子や、有用な糖タンパク質の生産を行なうための遺伝子を複数個導入することは困難である。前述のように、哺乳類型の糖

鎖を生産するのに必要な糖転移酵素群は十数個あることが知られており、この酵母細胞を宿主とすることは糖鎖構造を思い通りに改変制御するためには不適であると考えられる。

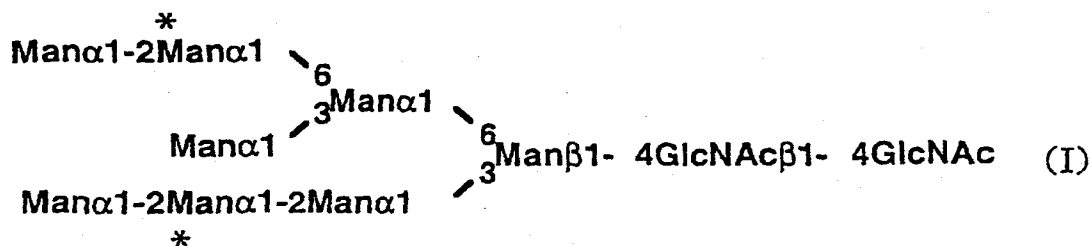
本発明の課題は、Asn-結合型糖タンパク質の酵母での生産における上記の問題を克服し、ヒトおよび他の哺乳類細胞の生産するハイマンノース型、混成型、複合型と同一の糖鎖構造を持つ糖鎖、および該糖鎖を含有する糖タンパク質を酵母を用いて製造する方法を提供することにある。

発明の開示

本発明者らは上記課題を解決すべく鋭意研究を重ねた結果、選択マーカーである栄養要求性変異を保持させつつ、即ち栄養要求性を相補する遺伝子を最終的に導入することなく、酵母に特異的な外糖鎖を生合成する遺伝子のうち、初発の延長付加反応を行なう α -1,6マンノシルトランスフェラーゼをコードする遺伝子（QCH1）、糖鎖の非還元末端にマンノースを付加する α -1,3マンノシルトランスフェラーゼをコードする遺伝子（MNN1）、およびマンノース-1-リン酸の付加を制御する遺伝子（MNN4）を破壊した新規な酵母変異株（栄養要求性三重変異株）によれば、哺乳類型糖鎖と同一の糖鎖構造を持つ糖鎖を生産できること、また該変異株に哺乳類型糖鎖の生合成系遺伝子を導入することで様々な哺乳類型糖鎖を生産できることを見い出した。

すなわち、本発明は、以下の(1)～(14)に関する。

(1) och1変異、mnn1変異、mnn4変異の変異形質と、少なくとも4個以上の栄養要求性変異形質を持つことを特徴とし、下記式(I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。＊はリ

ン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

(2) 栄養要求性を相補する遺伝子を最終的に導入することなく、OCH1遺伝子を破壊したoch1変異 ($\Delta och1$)、MNN1遺伝子を破壊したmnn1変異 ($\Delta mnn1$)、MNN4遺伝子を破壊したmnn4変異 ($\Delta mnn4$) の変異形質と、少なくとも1個以上の栄養要求性変異形質を有することを特徴とし、上記式 (I) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

(3) 栄養要求性変異形質が ura3変異、his3変異、leu2変異、ade2変異、trp1変異、can1変異から選ばれる、上記(1) または(2) に記載の酵母変異株。

(4) サッカロミセス (Saccharomyces) 属に属する酵母である、上記(3) に記載の酵母変異株。

(5) サッカロミセス・セレビシエ (Saccharomyces cerevisiae) に属する酵母である、上記(4) に記載の酵母変異株。

(6) サッカロミセス・セレビシエ (Saccharomyces cerevisiae) TIY19 株である、上記(5) に記載の酵母変異株。

(7) 上記(1) ~ (6) のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (I) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

(8) 上記(1) ~ (6) のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (I) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(9) 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた上記(1) ~ (6) のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (I) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タン

パク質を採取することを特徴とする、糖タンパク質の製造法。

(10) och1変異、mnn1変異、mnn4変異の変異形質を持つ酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも二つ以上導入した酵母変異株。

(11) 上記(1)～(6)のいずれかに記載の酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも一つ以上導入した酵母変異株。

(12) 上記(10) または (11)に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

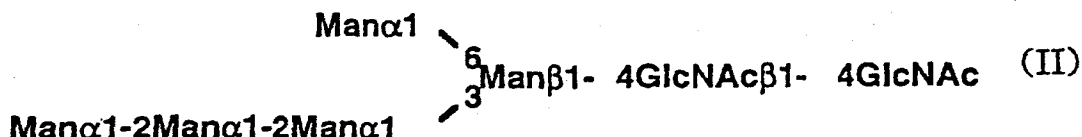
(13) 上記(10) または(11) に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(14) 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた上記(10) または(11) に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

本発明者らはまた、上記酵母 α -1,6マンノシルトランスフェラーゼをコードする遺伝子 (OCH1)、糖鎖の非還元末端にマンノースを付加する α -1,3マンノシルトランスフェラーゼをコードする遺伝子 (MNN1)、およびマンノース-1-リン酸の付加を制御する遺伝子 (MNN4) を破壊した上記酵母変異株 (栄養要求性三重変異株) に、更にERでの糖鎖生合成に関与する遺伝子 (ALG3) を破壊した新規な酵母変異株 (栄養要求性四重変異株) によれば、哺乳類型糖鎖の生合成系遺伝子の1つである α -mannosidase II遺伝子を導入することなく、その他の哺乳類型糖鎖の生合成系遺伝子を導入することにより、様々な哺乳類型糖鎖を生産できることを見い出した。

すなわち、本発明は、更に以下の (15) ~ (30) に関する。

(15) och1変異、mnn1変異、mnn4変異、alg3変異の変異形質と、少なくとも 5 個以上の栄養要求性変異形質を持つことを特徴とし、下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

(16) 栄養要求性を相補する遺伝子を最終的に導入することなく、OCH1遺伝子を破壊したoch1変異 (Δ och1)、MNN1遺伝子を破壊したmnn1変異 (Δ mnn1)、MNN4遺伝子を破壊したmnn4変異 (Δ mnn4)、ALG3遺伝子を破壊したalg3変異 (Δ alg3) の変異形質と、少なくとも 1 個以上の栄養要求性変異形質を持つことを特徴とし、上記式 (II) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

(17) 栄養要求性変異形質がura3変異、his3変異、leu2変異、ade2変異、trp1変異、can1変異から選ばれることを特徴とする、上記(15)または(16)に記載の酵母変異株。

(18) サッカロミセス (*Saccharomyces*) 属に属する酵母である、上記(17)に記載の酵母変異株。

(19) サッカロミセス・セレビシエ (*Saccharomyces cerevisiae*) に属する酵母である、上記(18)に記載の酵母変異株。

(20) サッカロミセス・セレビシエ (*Saccharomyces cerevisiae*) YS134-4A株である、上記(19)に記載の酵母変異株。

(21) 上記(15)~(20)のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (II) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

(22) 上記(15)～(20)のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (II) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(23) 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた上記(15)～(20)のいずれかに記載の酵母変異株を培地に培養し、培養物中に上記式 (II) で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(24) och1変異、mn1変異、mn4変異、alg3変異の変異形質を持つ酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも二つ以上導入した酵母変異株。

(25) 上記(15)～(20)のいずれかに記載の酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも一つ以上導入した酵母変異株。

(26) 上記(24)または(25)に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

(27) 上記(24)または(25)に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(28) 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた上記(24)または(25)に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

(29) α -マンノシダーゼII遺伝子が導入され、 α -マンノシダーゼII活性を有する酵母株。

(30) 上記(29) に記載の酵母株を培地に培養し、培養物中に生成蓄積された α -マンノシダーゼIIを採取することを特徴とする、 α -マンノシダーゼIIの製造法。

本明細書は本願の優先権の基礎である日本国特許出願平成11年第233215号の明細書及び／または図面に記載される内容を包含する。

図面の簡単な説明

図1は、哺乳類動物での一般的なN-結合型糖鎖の生合成経路を示す。

図2は、酵母 (*S. cerevisiae*) におけるN-結合型糖鎖の生合成経路を示す。図中、H、C、Eは、それぞれ図3のI、D、Fに続く。

図3は、酵母 (*S. cerevisiae*) におけるN-結合型糖鎖の生合成経路（続き）を示す。

図4は、従来の酵母遺伝子の破壊法を示す。

図5は、栄養要求性を相補する遺伝子を最終的に導入することなく、遺伝子を破壊する方法を示す。

図6は、TIY19株細胞表層マンナンタンパク質糖鎖の構造解析を示す。

図7は、 α -1,2-mannosidase遺伝子を導入したTIY19株の細胞表層マンナンタンパク質糖鎖のAmide-80カラムでの構造解析を示す。

a : TIY19株のマンナン糖タンパク質の糖鎖

b : α -1,2-mannosidaseを導入したTIY19株のマンナン糖タンパク質の糖鎖

図8は、 α -1,2-mannosidase遺伝子を導入したTIY19株の細胞表層マンナンタンパク質糖鎖のODS-80T_Mカラムでの構造解析を示す。

a : 式 (III) で示される構造の標準糖鎖

b : 図6で分取した画分

図9は、GnT-I活性測定の結果を示す。

図10は、 α -1,2-mannosidase遺伝子とGnT-I遺伝子を導入したTIY19株の細胞表層マンナンタンパク質糖鎖のAmide-80カラムでの構造解析を示す。

A : ベクターのみを導入したTIY19株の糖鎖構造解析

B : α -1,2-mannosidase遺伝子とGnT-1遺伝子を導入したTIY19株の糖鎖構造解析

a : Man₅GlcNAc₂-PA

b : GlcNAcMan₅GlcNAc₂-PA

c : Man₆GlcNAc₂-PA

d : Man₇GlcNAc₂-PA

e : Man₈GlcNAc₂-PA

図 1 1 は、 α -1,2-mannosidase 遺伝子と GnT-I 遺伝子を導入した TIY19 株の細胞表面マンナンタンパク質糖鎖の ODS-80T_M カラムでの構造解析を示す。

A : 標準品の混合物

B : 図 1 0 . B で分取した画分

図 1 2 は、 α -mannosidase II 遺伝子を導入した YPH500 株の細胞抽出液を用いたウエスタンブロット解析を示す。

A : ベクターのみ (pYEX-BX-3HA) を導入した YPH500 株からの細胞抽出液のウエスタンブロット解析結果

B : キメラ α -mannosidase-II 遺伝子を含むベクター (pYEOM2-HA) を導入した YPH500 株の細胞抽出液のウエスタンブロット解析結果

図 1 3 は、 α -mannosidase II 遺伝子を導入した YPH500 株の細胞抽出液を用いた α -mannosidase II 活性測定の結果を示す。

A : ベクターのみ (pYEX-BX-3HA) を導入した YPH500 株での活性測定結果

B : キメラ α -mannosidase-II 遺伝子を含むベクター (pYEOM2-HA) を導入した YPH500 株での活性測定結果

a : GlcNAcMan₅GlcNAc₂-PA

b : GlcNAcMan₃GlcNAc₂-PA

図 1 4 は、FGF 遺伝子を導入した TIY48 株 (上段)、及び FGF 遺伝子と α -1,2-mannosidase 遺伝子を導入した TIY53 株 (下段) の FGF 糖鎖の Amido-80 カラムでの構造解析を示す。

符号の説明

GlcNAc, GN : N- アセチルグルコサミン

Man, M : マンノース

PA : 2-アミノピリジル化

配列表の簡単な説明

- 配列番号 1 は、MNN1遺伝子の 5' 領域を増幅するためのプライマーA を示す。
- 配列番号 2 は、MNN1遺伝子の 5' 領域を増幅するためのプライマーB を示す。
- 配列番号 3 は、MNN1遺伝子の 3' 領域を増幅するためのプライマーC を示す。
- 配列番号 4 は、MNN1遺伝子の 3' 領域を増幅するためのプライマーD を示す。
- 配列番号 5 は、MNN4遺伝子の 3' 領域を増幅するためのプライマーE を示す。
- 配列番号 6 は、MNN4遺伝子の 3' 領域を増幅するためのプライマーF を示す。
- 配列番号 7 は、MNN4遺伝子の 5' 領域を増幅するためのプライマーG を示す。
- 配列番号 8 は、MNN4遺伝子の 5' 領域を増幅するためのプライマーH を示す。
- 配列番号 9 は、ALG3遺伝子の 5' 領域を増幅するためのプライマーI を示す。
- 配列番号 10 は、ALG3遺伝子の 5' 領域を増幅するためのプライマーJ を示す。
- 配列番号 11 は、ALG3遺伝子の 3' 領域を増幅するためのプライマーK を示す。
- 配列番号 12 は、ALG3遺伝子の 3' 領域を増幅するためのプライマーL を示す。
- 配列番号 13 は、 α -mannosidase II 遺伝子の N 末端側領域を増幅するためのプライマーM を示す。
- 配列番号 14 は、 α -mannosidase II 遺伝子の N 末端側領域を増幅するためのプライマーN を示す。
- 配列番号 15 は、 α -mannosidase II 遺伝子の中央部領域を増幅するためのプライマーO を示す。
- 配列番号 16 は、 α -mannosidase II 遺伝子の中央部領域を増幅するためのプライマーP を示す。
- 配列番号 17 は、 α -mannosidase II 遺伝子の C 末端側領域を増幅するためのプライマーQ を示す。
- 配列番号 18 は、 α -mannosidase II 遺伝子の C 末端側領域を増幅するためのプライマーR を示す。
- 配列番号 19 は、HA-tag を 3 回反復させるように結合させる遺伝子をコードする二本鎖DNAの配列S を示す。
- 配列番号 20 は、OCH1遺伝子の膜貫通領域をコードする二本鎖DNAの配列T

を示す。

配列番号 21 は、 α -mannosidase II 遺伝子の触媒領域の一部を増幅するためのプライマー U を示す。

配列番号 22 は、 α -mannosidase II 遺伝子の触媒領域の一部を増幅するためのプライマー V を示す。

配列番号 23 は、ヒト UDP-GlcNAc Transporter 遺伝子を増幅するためのプライマー W を示す。

配列番号 24 は、ヒト UDP-GlcNAc Transporter 遺伝子を増幅するためのプライマー X を示す。

配列番号 25 は、ヒト prepro α -factor と FGF 遺伝子を増幅するためのプライマー Y を示す。

配列番号 26 は、ヒト prepro α -factor と FGF 遺伝子を増幅するためのプライマー Z を示す。

発明を実施するための形態

以下、本発明を詳細に説明する。

本発明の酵母変異株に必要な変異形質は、酵母特有の外糖鎖生合成系遺伝子の変異であり、具体的には och1 変異、mnn1 変異、mnn4 変異、あるいは och1 変異、mnn1 変異、mnn4 変異、alg3 変異である。

即ち、上記の変異を有する限り、自然変異株であっても人為変異株であってもよい。

また、本発明の酵母変異株における外来遺伝子を導入するための栄養要求性変異形質は、使用する酵母株に規定されるものであり、具体的には ura3 変異、his3 変異、leu2 変異、ade2 変異、trp1 変異、can1 変異から選ばれる。栄養要求性変異形質の数は導入する遺伝子の数によるが、一般的に、1つの遺伝子を導入するのに1個の栄養要求性変異形質が必要である。複数の遺伝子を導入する場合は、導入する遺伝子断片が長く、導入効率が低下し、ひいては発現効率も低下するので、導入遺伝子の数が多いほど多数の栄養要求性変異形質が必要となる。

本発明において、「栄養要求性を相補する遺伝子」とは、アミノ酸、核酸等の

生体成分の合成系の遺伝子である。変異形質はこれらの遺伝子が機能しないような変異が入っているものなので、相補する遺伝子は元の機能する遺伝子そのものである。よって、元の酵母株由来の遺伝子が望ましい。

また、「栄養要求性を相補する遺伝子を最終的に導入することなく」とは、一つまたはそれ以上の遺伝子を破壊する（変異形質を導入する）のに1個またはそれ以上の選択マーカー、即ち、栄養要求性変異形質を利用するが、破壊後も破壊遺伝子数と同数の当該形質が残っており、再度の遺伝子破壊の際に繰り返し同じ当該形質を使用できることをいう（図5参照）。

本発明における、外来遺伝子を導入するための栄養要求性変異形質を保持し、かつ酵母特有の外糖鎖生合成系遺伝子が破壊された酵母変異株（以下、栄養要求性変異株）は以下のようにして作製することができる。

まず、標的遺伝子の破壊に必要なDNA遺伝子断片の単離は、Saccharomyces cerevisiaeのゲノムプロジェクトにより、いずれもその染色体上の配座が既知である（Goffeau et al., Nature, 387 (suppl.), 1-105 (1997)) ことから、米国ATCC (American Type Culture Collection) など公的な機関から標的遺伝子の近傍を含む遺伝子断片の分与を受けることが可能である（ATCC Recombinant DNA materials, 3rd edition, 1993）。また、一般的手法にしたがってS. cerevisiae からゲノムDNAを抽出し、目的遺伝子を選別することにより可能である。上記において、S. cerevisiaeからゲノムDNAの抽出は、例えば、Cryerらの方法（Methods in Cell Biology, 12, 39-44 (1975)）およびP. Philippsenらの方法（Methods Enzymol., 194, 169-182 (1991)）に従って行なうことができる。

標的遺伝子は、PCR法により増幅させてから遺伝子破壊を行なう。PCR法は、インビトロ (*in vitro*)でDNAの特定断片をその領域の両端のセンス・アンチセンスプライマー、耐熱性DNAポリメラーゼ、DNA増幅システム等の組み合わせを用いて約2～3時間で数十万倍以上に増幅できる技術であるが、標的遺伝子の増幅には、プライマーとして25～30merの合成1本鎖DNAを、鋳型としてゲノムDNAを用いる。

本発明において標的遺伝子の破壊は、Rothstein, Methods Enzymol., 101,

202-211 (1983)に開示される方法に基本的に従って行いうる。本方法は、まず、プラスミド上の標的遺伝子DNAを分断あるいは部分欠失させ、そこに適当な選択マーカ―遺伝子DNAを挿入して標的遺伝子の上流部と下流部の間に選択マーカ―がサンドイッチされた構造体を作製し、次に、この構造体を酵母細胞に導入する。以上の操作により、導入断片（選択マーカ―を挟み込んだDNA構造体）の両端と染色体上の標的遺伝子との相同部分の間で2回の組み換えを起こし、染色体上の標的遺伝子が導入断片で置換される。

具体的に、MNN1遺伝子破壊株の作製を例にとり説明する。Alaniらによって構築された、サルモネラ菌のhisG遺伝子DNA断片がURA3遺伝子の両端に結合されているプラスミド (Alani et al., Genetics, 116, 541-545 (1987))からhisG-URA3-hisGのカセットを制限酵素で切りだし、プラスミド上の標的遺伝子に挿入し、破壊された対立遺伝子を構築する。このプラスミドを用いて染色体の標的遺伝子と置換して遺伝子破壊株を得る。染色体に挿入されたURA3遺伝子はhisGではさまれており、hisG配列間での相同的組み換えにより1コピーのhisGとともに染色体から脱落する。染色体上の標的遺伝子にはなおも1コピーのhisG断片が残り破壊されたままであるが、宿主細胞はUra⁻表現形となる（図5）。このhisG間での相同的組み換えは5-フルオロオロト酸（5-FOA）により行なうことができる。ura3変異株は5-FOAに耐性であり (Boeke et al., Mol. Gen. Genet., 197, 345-346 (1984); Boeke et al., Methods Enzymol., 154, 165-174 (1987))、Ura³⁺表現形を持つ細胞株は5-FOA培地に生育できなくなる。よって、5-FOAを加えた培地で耐性形質を持つ株を分離すれば、再びURA3を用いての操作が可能である。

以下、このMNN1遺伝子破壊株に、同様な手法にてMNN4遺伝子破壊、OCH1遺伝子破壊を行うことにより、本発明の目的とする栄養要求性三重変異株（Δoch1Δmnn1Δmnn4）を得ることができる。また更に同様な手法にてALG3遺伝子破壊を行うことにより、本発明の目的とする栄養要求性四重変異株（Δoch1Δmnn1Δmnn4Δalg3）を得ることができる。

従って、上記の手法により人為的に遺伝子破壊を行った「人為破壊株」では、元の酵母株を有する栄養要求性変異形質が遺伝子破壊操作により損なわれない。

従って、該人為変異株の有する栄養要求性変異形質の数は、三重変異株であっても四重変異株であっても元の酵母株が有する栄養要求性変異形質の数に等しく、少なくとも1個以上有することになる。

一方、上記の手法によらず遺伝子破壊が自然に起こっている「自然変異株」では、上記の手法を用いることはないので栄養要求性変異形質の数の増減とは関係がない。

本発明におけるM8ハイマンノース糖鎖を生産する酵母変異株を作成する場合、従来法に基づきOCH1、MNN1、MNN4遺伝子を破壊するのに6個の栄養要求性変異形質を保有する酵母株を用いて作成すると、栄養要求性変異形質は3個しか残らないので、該変異株の有する栄養要求性変異形質の数は少なくとも4個以上となる。

また、OCH1、MNN1、MNN4遺伝子変異に更にALG3遺伝子に変異が生じたM8ハイマンノース糖鎖を生産する酵母変異株を作成する場合、従来法による作成ではmnn1変異、alg3変異の自然変異株を利用できるが、更にOCH1、MNN4遺伝子を破壊する必要があるため2個の栄養要求性変異形質を使用することになる。よって、上記の6個の栄養要求性変異形質を保有する酵母株を用いると、栄養要求性変異形質は4個しか残らないので該変異株の栄養要求性変異形質の数は少なくとも5個以上となる。

なお、上記操作で選択マーカーとしては、URA3などの栄養要求性マーカーのみならず、G418、セルレニン、オーレオバシジン、ゼオシン、カナバニン、シクロヘキシミド、テトラサイクリン等の薬剤に対し、耐性を付与するマーカーなども使用できる。また、メタノールやエタノールなどに対する溶剤耐性や、グリセロールや塩などに対する浸透圧耐性、銅などの金属イオン耐性等を付与する遺伝子をマーカーにすることで、遺伝子の導入や破壊を行うことも可能である。

上記操作における、DNAの細胞への導入およびこれによる形質転換の方法としては、一般的な方法、例えば、ベクターとしてファージを用いる場合は、大腸菌宿主にこれを感染させる方法等により、効率よく宿主にDNAを取り込ませることができる。またプラスミドを用いて酵母を形質転換する方法としては、リチウム塩で処理して自然にDNAを取込みやすい状態にしてプラスミドを取り込ま

せる方法や、あるいは電氣的にDNAを細胞内に導入する方法を採用できる (Becker and Guarente, *Methods Enzymol.*, 194, 182-187 (1991))。

また、上記操作における、DNAの単離・精製等は何れも常法、例えば大腸菌の場合、アルカリ/SDS法とエタノール沈殿によるDNA抽出、更にRNase処理、PEG沈殿法などによりDNAを精製できる。また、遺伝子のDNA配列の決定等も通常の方法、例えばジデオキシ法 (Sanger et al., *Proc. Natl. Acad. Sci., USA*, 74, 5463-5467 (1977)) 等により行なうことができる。更に上記DNA塩基配列の決定は、市販のシーケンスキット等を用いることによっても容易に行ない得る。

以上のようにして作製された栄養要求性変異株は、ハイマンノース型の哺乳類型糖鎖を生産することができるが、さらにハイブリッド型、複合型の糖鎖哺乳類型糖鎖を生産させるためには、該変異株に酵母特有の糖鎖加水分解酵素遺伝子群、糖転移酵素遺伝子群を導入する。また、本来糖鎖の生合成は前述のようにER、ゴルジ体内で行われるので、糖鎖の原料となる糖ヌクレオチドがこれらの器官に存在することが必要であるが、酵母内ではこれらの糖ヌクレオチド輸送体はないか、有っても実際に糖鎖が生合成される器官には微量しか存在しない。従って、細胞質内で生合成された糖ヌクレオチドを細胞質からER、ゴルジ体内へ移動させる糖ヌクレオチド輸送体遺伝子群が更に必要である。

従って、本発明においては、上記の糖鎖加水分解酵素遺伝子群、糖転移酵素遺伝子群、糖ヌクレオチド輸送体遺伝子群に属する遺伝子を「哺乳類型糖鎖の生合成系遺伝子」という。

糖鎖加水分解酵素遺伝子群としては、 α -マンノシダーゼ (α -mannosidase I, α -mannosidase II)等の遺伝子、糖転移酵素遺伝子群としては、N-アセチルグルコサミニルトランスフェラーゼ (GnT-I, GnT-II, GnT-III, GnT-IV, GnT-V)、ガラクトシルトランスフェラーゼ (GalT)、フコシルトランスフェラーゼ (FucT)等の遺伝子、糖ヌクレオチド輸送体遺伝子群としては、UDP-GlcNAc Transporter, UDP-Gal Transporter 等の遺伝子が挙げられる。これらの遺伝子は哺乳類由来の遺伝子を単離して利用することもできるし、また遺伝子を合成することによっても可能である。

上記の「哺乳類型糖鎖の生合成系遺伝子」は、上記の1種または2種以上の遺

伝子群に属する遺伝子を、目的とする糖鎖を生産するのに必要な数だけ導入する。導入する遺伝子が複数の場合は、それらの遺伝子が同種の遺伝子群に属していても、互いに異種の遺伝子群に属していてもよい。

上記の栄養要求性変異株、あるいは、該栄養要求性変異株に上記の外来遺伝子群を導入した変異株を、培地に培養すれば、酵母特有の外糖鎖の含量が低下し、哺乳類細胞の生産するハイマンノース型糖鎖 ($\text{Man}_5\text{-}\beta\text{-GlcNAc}_2$)、混成型糖鎖 ($\text{GlcNAcMan}_5\text{GlcNAc}_2$)、複合型糖鎖 ($\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ 等) と同一の Asn 結合型糖鎖を含有する糖タンパク質を、酵母細胞内または細胞外に生産させることができる。

具体的には、栄養要求性変異株として三重変異株 ($\Delta\text{och1}\Delta\text{mnn1}\Delta\text{mnn4}$) を用いる場合は、該変異株に α -mannosidase I 遺伝子と GnT-I 遺伝子を導入することにより、混成型糖鎖を生産させることができ、また、哺乳類型糖鎖の生合成系遺伝子 (α -mannosidase II、GnT-II、GalT、UDP-GlcNAc Transporter、UDP-Gal Transporter 遺伝子) を導入することにより、二本鎖複合型糖鎖 ($\text{Gal}_2\text{GlcNAc}_2\text{Man}_2\text{GlcNAc}_2$) を生産させることができる。

さらに、GnT-IV、GnT-V 遺伝子を導入することにより、三本鎖複合型糖鎖、四本鎖複合型糖鎖を生産させることもできる。

また、栄養要求性変異株として四重変異株 ($\Delta\text{och1}\Delta\text{mnn1}\Delta\text{mnn4}\Delta\text{alg3}$) を用いる場合は、 α -mannosidase II 遺伝子を導入することなく、哺乳類型糖鎖の生合成系遺伝子 (α -mannosidase I、GnT-I、GnT-II、Gal-T、UDP-GlcNAc Transporter、UDP-Gal Transporter 遺伝子) を導入することにより、二本鎖複合型糖鎖 ($\text{Gal}_2\text{GlcNAc}_2\text{Man}_2\text{GlcNAc}_2$) を生産させることができる。

生成する糖鎖、及び糖タンパク質を高収率で得るためには、上記酵素を適切な器官 (例えばゴルジ体) にて高発現をさせることが望ましい。よって酵母のコードン使用頻度に合わせた遺伝子を用いることは有効である。また酵素を適切な器官に局在させるためには、酵母のシグナル配列等を付加することも有効である。遺伝子の導入については $2\mu\text{m}$ プラスミドタイプ (YEp タイプ)、染色体組み込み型タイプ (YIp タイプ) 等ベクターを用いる方法が考えられるが、目的に応じて使い分けることができる。YEp タイプのベクターは遺伝子を多コピーで導入でき

るので、遺伝子を大量に発現させることができる。一方、YIpタイプのベクターは遺伝子を染色体に存在させることができるためその遺伝子を安定的に保持することができる。遺伝子を発現させるために必要プロモーターはGAPDH, PGK等の構成的発現プロモーター、GAL1, CUP1等の誘導発現プロモーターなど特に限定されないが、糖鎖の生産には構成的発現プロモーターが望ましい。しかし糖鎖加水分解酵素、糖転移酵素、糖ヌクレオチド輸送体遺伝子を1種または複数発現させた場合には酵母の増殖に影響を及ぼすことがあるので、その場合には誘導プロモーターの使用や、遺伝子を導入する順序を考慮する必要がある。

また、本発明における栄養要求性変異株には、上記の如く人為的な遺伝子破壊法によって得られる変異株以外に、薬剤や紫外線照射、自然変異により得られる変異株も含まれる。この自然変異株も同様に、前述の哺乳類型糖鎖の生合成系遺伝子（糖鎖加水分解酵素遺伝子群、糖転移酵素遺伝子群、糖ヌクレオチド輸送体群に属する遺伝子）を導入することにより、哺乳類型糖鎖や哺乳類型糖鎖を有する糖タンパク質の生産を行うことが可能である。

更に、上記の糖鎖を持つ異種生物由来の糖タンパク質を生産させるためには、上記酵母変異株を宿主として、目的の糖タンパク質をコードする遺伝子（cDNAなど）を酵母で発現出来るプロモーターの下流に接続した遺伝子を作製し、相同組換えによって上記酵母宿主に組み込むか、或いは、プラスミドに挿入して上記宿主を形質転換することにより、上記宿主の形質転換体を作製し、これを公知の方法により培養することにより、酵母細胞内または細胞外に生産された目的の糖タンパク質を回収できる。

上記の酵母変異株の培養は、酵母の培養に慣用される常法に従って行なうことができる。例えば、Difco社から供給される各種の培地成分を添加し、かつプラスミドの複製・保持に必要なマーカーによって供給可能となるアミノ酸を除いた合成培地（炭素源、窒素源、無機塩類、アミノ酸、ビタミン等を含む）等を利用できる（Sherman, Methods Enzymol., 194, 3-57 (1991)）。

上記の培養物（培養液、培養菌体）から糖タンパク質を単離精製するためには、通常のタンパク質の単離、精製法を用いればよい。

例えば、培養終了後、細胞を遠心分離により回収し水系緩衝液にけん濁後、超

音波破碎機、フレンチプレス、マントンガウリンホモゲナイザー、ダイノミル等により細胞を破碎し、無細胞抽出液を得る。該無細胞抽出液を遠心分離することにより得られた上清から、通常のタンパク質の単離精製法、即ち、溶媒抽出法、硫酸等による塩析法、脱塩法、有機溶媒による沈殿法、ジエチルアミノエチル (DEAE) セファロース等レジンを用いた陰イオン交換クロマトグラフィー法、S-Sepharose FF (ファルマシア社製) 等のレジンを用いた陽イオン交換クロマトグラフィー法、ブチルセファロース、フェニルセファロース等のレジンを用いた疎水性クロマトグラフィー法、分子篩を用いたゲルろ過法、His Bindレジン (Novagen社製) などを用いたアフィニティークロマトグラフィー法、クロマトフォーカシング法、等電点電気泳動等の電気泳動法等の手法を単独あるいは組み合わせて用い、精製標品を得ることができる。

実施例

以下、実施例により本発明を具体的に説明する。ただし、これらの実施例は本発明の技術的範囲を何等限定するものではない。

〔実施例 1〕 哺乳類型糖鎖生産能を有する酵母変異株 ($\Delta mnn1 \Delta mnn4 \Delta och1$ 栄養要求性三重変異株) の育種

(1) $\Delta mnn1$ 栄養要求性変異株の作製とその性質

すでに報告のあるpNK51(Alani et al., Genetics, 116, 541-545 (1987))より、URA3遺伝子の両端にサルモネラ菌hisG遺伝子がダイレクトリピートで結合しているカセット (HUH) をBglIIとBamHIで切断し、大腸菌プラスミドpSP73のBamHI部位に挿入した。このプラスミドをpSP73-HUHと命名した。

MNN1遺伝子は、酵母第5番染色体動原体近傍に位置し、MNN1遺伝子のDNA塩基配列は、GenBankデータベースにL23753で登録されている(Yip et al., Proc. Natl. Acad. Sci. USA, 9, 2723-2727 (1994))。 MNN1遺伝子の5'領域をプライマーA(GGATCCGAAGAAAACCTAATACATTGAAGT : 配列番号1)とプライマーB(GCATGCCCTTTGGTTTAATATAAATCTCCGGAGTGC : 配列番号2)を用いて、また、3'領域をプライマー

C(GCATGCTACATAACTCCAATCAGCAGCAAATATGTC : 配列番号 3) とプライマーD(GCGGCCGCGTGTCTGTTCGGGTAACGTTTAAACCAAT : 配列番号 4) を用いて、それぞれPCRで増幅した。これらのDNA断片をHIS3マーカを持つプラスミドpHYHのSphI部位に組み込み、pHYH Δ mnn1を作製した。MNN1遺伝子をHUHカセットを用いて破壊するために、1.8KbのSphI断片をpHYH Δ mnn1より取得し、pSP73-HUHのSphI部位に挿入したpSP73- Δ mnn1::HUHを構築した。本プラスミドをNotI部位で切断することにより直鎖化し、野生株W303-1A(MATa leu2-3, 112his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100)を酢酸リチウム法 (Ito et al., J. Bacteriol., 153, 163-168 (1983)) を用いて形質転換した。形質転換後、SD-Ura (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ウラシルを除く核酸塩基、およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。

形質転換体よりゲノムDNAを調製し、PCR法によりウラシルマーカがMNN1領域の染色体に組み込まれていることを確認し、TIY1株とした。

本株を、5-FOAを含有したYSD 培地 (1%酵母抽出液、2%グルコース、アデニン (40 mg/L)、ウラシル(20mg/L)) にて選抜を行ない、URA3遺伝子脱落株を得た。上記の方法と同様にPCR法を用いてURA3遺伝子を脱落させたmnn1破壊株を確認した。 Δ mnn1::hisGを含む本株をTIY3株とした。

MNN1破壊株は、非還元末端の α -1,3結合のマンノースを欠損しているため、N-結合型修飾をうけるインベルターゼの移動度が、野生株より早くなることが知られている。YPAD培地にて培養した野生株およびTIY3株をそれぞれシュクロース0.2%を含む栄養培地 (1%酵母抽出液、2% Bactoペプトン、アデニン (40 mg/L)) に再懸濁し、3時間培養した。菌体を回収したのち、SDSサンプルバッファーに懸濁しガラスビーズにて粉碎したのち、上清を用いて6 % SDSポリアクリルアミド電気泳動を行った。インベルターゼの検出はシュクロースで誘導後、トリフェニルテトラゾリウムを用いて活性染色を行った (Ballou, Methods Enzymol., 185, 440-470 (1990))。その結果、TIY3株の生産するインベルターゼは、野生株のものより移動度が早いことを確認した。

(2) $\Delta mnn1 \Delta mnn4$ 栄養要求性二重変異株の作製とその性質

MNN4遺伝子は、酵母第11番染色体に位置し、MNN4遺伝子のDNA塩基配列は、GenBankデータベースにD83006で登録されている(Odani et al., Glycobiology, 6, 805-810 (1996))。 MNN4遺伝子の3'領域をプライマーE (AGATGCATACTAGTGGGCCCATTGTGATTGGAAT : 配列番号5) とプライマーF (CCCCCGAATTCGTGTGAAGGAATAGTGACG : 配列番号6) を用いて、また、5'領域をプライマーG (CCCCCGAATTCAAGTCGGAGAACCTGACTG : 配列番号7) とプライマーH (ATGGGCCCCTAGTATGCATCTCGCGTGGCATGG : 配列番号8) を用いて、それぞれPCRで増幅した。これらのDNA断片をHUHカセットを持つ前記 pSP73-HUHの EcoRI部位に組み込み、pSP73- $\Delta mnn4::HUH$ を作製した。本プラスミドを SpeI部位で切断することにより直鎖化し、TIY3株を酢酸リチウム法を用いて形質転換した。形質転換後、SD-Ura培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。

形質転換体よりゲノムDNAを調製し、PCR法によりウラシルマーカが MNN4領域の染色体に組み込まれていることを確認し、TIY9株とした。

本株を、5-FOAを含有したYSD 培地にて選抜を行ない、URA3遺伝子脱落株を得た。上記の方法と同様にPCR法を用いてURA3遺伝子を脱落させた mnn4破壊株を確認した。 $\Delta mnn1::hisG \Delta mnn4::hisG$ を含む本株をTIY11株とした。

糖鎖中のリン酸基の有無は、アルシアンブルー(alcian blue)で染め分けることができる。アルシアンブルーは正に荷電しており、負の荷電と結合する色素である。そこで、酵母細胞をpH3の緩衝液に懸濁して0.1%のアルシアンブルー8GX(Sigma製、code No. A3157)を加えると、糖鎖中にリン酸基をもつものだけが青く染まり、リン酸基をもたないものは白くなる。 $\Delta mnn4$ 破壊株は、細胞表層の糖鎖がほとんどリン酸基を持たないため、アルシアンブルーに染まらない。野生株、TIY3およびTIY11を栄養培地にて培養し、それぞれの細胞をアルシアンブルーにより染色した結果、TIY11のみ青く染まらないことを確認した。

(3) $\Delta mnn1 \Delta mnn4 \Delta och1$ 栄養要求性三重変異株の作製とその性質

OCH1遺伝子は、酵母第7番染色体に位置し、OCH1遺伝子のDNA塩基配列は、

GenBankデータベースにD11095で登録されている (Nakayama et al., EMBO J., 11, 2511-2519 (1992))。すでに構築されているOCH1遺伝子の全長を含む pBL-OCH1 (Nakayama et al., EMBO J., 11, 2511-2519 (1992)) のOCH1遺伝子内部 AatII-HpaI 部位を切断し、平滑末端化した pNKY51 より得た HUH カセットを挿入した pBL- $\Delta och1::HUH$ を作製した。本プラスミドを SalI および BamHI で切断することにより $\Delta och1::HUH$ を含む領域を切り出し、TIY11 を酢酸リチウム法で形質転換した。 $\Delta och1$ 破壊を含む株は低浸透圧感受性を示すため、形質転換後、0.3 M KCl を含む SD-Ura 培地のプレートにまいて、30°C で 2 日間培養し、形質転換体を得た。

形質転換体よりゲノム DNA を調製し、PCR 法によりウラシルマーカが Δ OCH1 領域の染色体に組込まれていることを確認し、TIY17 株とした。

本株を、5-FOA と 0.3 M KCl を含む YSD 培地にて選抜を行ない、URA3 遺伝子脱落株を得た。上記の方法と同様に PCR 法を用いて URA3 遺伝子を脱落させた och1 破壊株を確認した。 $\Delta mnn1::hisG$ $\Delta mnn4::hisG$ $\Delta och1::hisG$ を含む本株を TIY19 株とした。

この栄養要求性三重変異株 TIY19 株は、工業技術院生命工学工業技術研究所 (茨城県つくば市東 1 丁目 1 番 3 号) に平成 11 年 7 月 27 日付で受託番号 FERM BP-6802 として国際寄託されている。

och1 破壊を含む本 TIY19 株は、ハイマンノース型糖鎖を形成するためインペルターゼの移動度が、野生株、TIY3 株、TIY11 株に比べ早くなることが知られている。そこで、och1 破壊株の糖鎖長における効果を確認するため、それぞれ野生株、TIY3, TIY11, TIY19 株より上記に示した方法でインペルターゼを検出した結果、野生株、TIY3, TIY11, TIY19 の順に移動度が早くなっていることを確認した。

〔実施例 2〕 哺乳類型糖鎖生産能を有する酵母変異株 ($\Delta mnn1 \Delta mnn4 \Delta och1 \Delta alg3$ 栄養要求性四重変異株) の作製とその性質

ALG3 遺伝子は、酵母第 2 番染色体に位置し、ALG3 遺伝子の DNA 塩基配列は、GenBank データベースに Z35844 で登録されている (Feldmann et al., EMBO J.,

13, 5795-5809 (1994))。 ALG3 遺伝子の 5' 領域をプライマー I (GCGGCCGCGAGACCTGAATCTTCGACACGCAAGAAAAA : 配列番号 9) と J (GAATTCGCTTTCGAACAAAATCAAAAGGGGCATAAC : 配列番号 10) を用いて、また、3' 領域をプライマー K (GAATTCCTATCCACCAAACCTCACAAGCAAGCA : 配列番号 11) と L (GCGGCCGCCGAGAGGGTGAACGGTGCTAACTCAGGATT : 配列番号 12) を用いて、それぞれ PCR で増幅した。これらの DNA 断片を HUH カセットを持つ pSP73-HUH の EcoRI 部位に組み込み、pSP73-alg3::HUH を作製した。本プラスミドを NotI 部位で切断することにより直鎖化し、TIY19 株を酢酸リチウム法を用いて形質転換した。形質転換後、SD-Ura 培地のプレートにまいて、30℃ で 2 日間培養し、形質転換体を得た。

形質転換体よりゲノム DNA を調製し、PCR 法によりウラシルマーカが ALG3 領域の染色体に組み込まれていることを確認し、YS134 株とした。

本株を、5-FOA を含有した SD 培地にて選抜を行ない、URA3 遺伝子脱落株を得た。上記の方法と同様に PCR 法を用いて URA3 遺伝子を脱落させた alg3 破壊株を確認した。 Δ mnn1::hisG Δ mnn4::hisG Δ och1::hisG Δ alg3::hisG を含む本株を YS134-4A 株とした。

この栄養要求性四重変異株 YS134-4A 株は、工業技術院生命工学工業技術研究所（茨城県つくば市東 1 丁目 1 番 3 号）に平成 11 年 7 月 27 日付で受託番号 FERM BP-6801 として国際寄託されている。

alg3 破壊を含む本 YS134-4A 株は糖鎖長が短いため、インペルターゼの移動度が、野生株、TIY3 株、TIY11 株、TIY19 株に比べ早くなることが知られている。そこで、alg3 破壊株の糖鎖長における効果を確認するため、それぞれ野生株、TIY3 株、TIY11 株、TIY19 株、YS134-4A 株より実施例 1 (1) に示した方法でインペルターゼを検出した結果、野生株、TIY3 株、TIY11 株、TIY19 株、YS134-4A 株の順に移動度が早くなっていることを確認した。

〔実施例 3〕 Δ mnn1 Δ mnn4 Δ och1 栄養要求性三重変異株からの細胞表層マナンタンパク質の分離とその含有糖鎖の構造解析

コンカナバリンAはC-3, C-4, C-6位の水酸基が未置換の α -D-Manを、2残基以上含む糖鎖に対して親和性を示すレクチンであり、これをカラムに固定することで、酵母細胞壁多糖であるグルカンやキチンなどとマンナンタンパク質を分離することができる。まず、TIY19株の菌体より細胞表層のマンナンタンパク質を分離した (Peat et al., J. Chem. Soc., 29 (1961))。

0.3 M KClを含むYPAD培地50 mlを500 ml容坂口フラスコに入れ、30℃で24時間培養し、菌体を遠心分離によって集め、10 mlの100 mMクエン酸ナトリウム緩衝液 (pH 7.0) に懸濁し、オートクレーブ中で121℃、1時間加熱した。冷却後、遠心分離し、上清を取り、固形物は、もう一度10 mlの水を加えて同様に加熱、遠心分離し、上清を集めた。全抽出液を合わせて、3倍量のエタノール中に注加した。生じた白色の沈殿物を乾燥させた。これをコンカナバリンA (ConA) カラム用緩衝液 (0.15 M塩化ナトリウム、0.5 mM塩化カルシウムを含む0.1 M リン酸ナトリウム緩衝液 (pH 7.2)) に溶解し、ConA-アガロースカラム (0.6 x 2 cm、ホーネンコーポレーション社製) に供し、ConAカラム用緩衝液で洗浄後、0.2 Mの α -メチルマンノシドを含むConAカラム用緩衝液で溶出を行なった。得られた画分を透析し、凍結乾燥を行なってマンナンタンパク質を得た。

次に得られたマンナンタンパク質に対し、酵素処理を施しAsn結合型糖鎖を切りだした。すなわち、凍結乾燥標品を100 μ lのN-グリコシダーゼF用緩衝液 (0.5% SDS, 0.35% 2-メルカプトエタノールを含む0.1 M Tris-HCl緩衝液 (pH8.0)) に溶解し、5分間煮沸処理をした。室温まで戻した後、50 μ lの7.5% Nonidet P-40、138 μ lのH₂O、12 μ lのN-glycosidase F (ベーリンガー社製) を加え、37℃、16時間処理した。BioRad AG501-X8カラムで脱塩後、等量のフェノール：クロロホルム (1:1) を加え激しく振盪して、界面活性剤とタンパク質を除去し、糖鎖調製品とした。

得られた糖鎖を蛍光標識 (ピリジルアミノ化、PA化という) するため、以下の操作を行なった。糖鎖調製品を濃縮乾固後、40 μ lのカップリング試薬 (552 mgの2-アミノピリジンを200 μ lの酢酸に溶解した) を加え、密封し、90℃、60分処理した。室温まで戻した後、140 μ lの還元試薬 (200 mgのボラン・ジメチ

ルアミン複合体を50 μ l のH₂Oと80 μ lの酢酸に溶解した)を加え、密封し、80℃、80分処理した。反応後、アンモニア水を200 ml加えた後、さらに等量になるようにフェノール：クロロホルム(1：1)を加え、激しく振盪してPA化オリゴ糖を含む水層を回収した。これを7回繰り返して、未反応の2-アミノピリジンを除去した。上清について0.22 μ mのフィルターで濾過し、PA化オリゴ糖調製品とした。

アミドカラムを用いたHPLCでは、PA化オリゴ糖をその鎖長によって分離することが可能である。カラムはTSKGel Amide-80 (4.6 x 250 mm、東ソー製)を使用し、溶媒は、200 mM酢酸-トリエチルアミン緩衝液(pH 7.0)とアセトニトリルとの35：65の混合液(A液)、200 mM酢酸-トリエチルアミン緩衝液(pH7.0)とアセトニトリルとの50：50の混合液(B液)を調製した。

予め溶媒Aを流速1.0 ml/minで流すことによりカラムを平衡化し、試料注入直後から溶媒Bの割合を25分かけて50%まで直線的に上昇させ、その後、溶媒Aと溶媒Bを50：50のまま5分間流し、PA化オリゴ糖を溶出した。その結果を図6に示す。 $\Delta och1 \Delta mnn1 \Delta mnn4$ 栄養要求性三重変異株であるTIY19株の生産するマンナンタンパク質の糖鎖は、アミドカラムでは主に1つのピークであった。このピークはMan₈GlcNAc₂-PA標品(宝酒造製)の溶出位置と一致した。したがって、TIY19株の生産するマンナンタンパク質にはMan₈GlcNAc₂のハイマンノース型糖鎖が付加していることが明らかとなった。

[実施例4] $\Delta och1 \Delta mnn1 \Delta mnn4$ 栄養要求性三重変異株への α -mannosidase I

遺伝子の導入

より哺乳類型に近い糖鎖を酵母で生合成するには、その初発反応を司る α -mannosidase I (α -1,2-mannosidase)の遺伝子を栄養要求性三重変異株に導入し、発現させればよい。これによりハイマンノース型糖鎖がさらに短くなった、哺乳類混成型または哺乳類複合型の前駆体であるMan₅GlcNAc₂糖鎖を生合成できる。

既に発現実績のあるAspergillus saitoi由来の α -1,2-mannosidaseの小胞体型

発現プラスミドpGAMH1 (Chiba et al., J. Biol. Chem., 273, 26298-26304 (1998)) を使い、TIY19株を酢酸リチウム法で形質転換した。コントロールとして、 α -1,2-mannosidase遺伝子を含まないベクターpG3だけで形質転換したものを用いた。形質転換後、SD-Trp (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、トリプトファンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。得られた形質転換体をTIY19pGAMH1 とした。

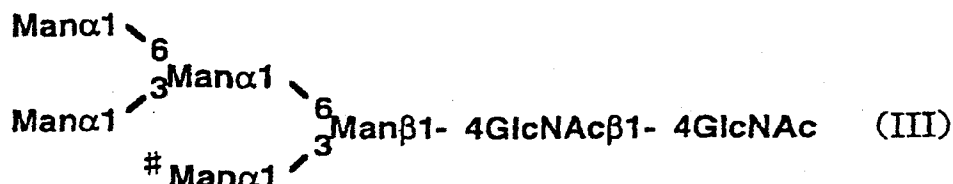
得られた形質転換体から実施例3と同様に糖鎖を調製、HPLCでの分析を行った。

アミドカラムによる分析結果を図7に示した。コントロールのベクターのみのものでは、実施例3の結果と同様、主に1つのピークであり (図7, a)、 $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ 標品 (宝酒造製) の溶出位置と一致した。一方、 α -1,2-mannosidase遺伝子を含むTIY19pGAMH1 では、主に4つのピークが見られた (図7, b)。これらのピークは溶出の早い順に $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_6\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_7\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ 標品と溶出位置が一致した。これらはヒト型のハイマンノース型糖鎖と呼ばれるものである。

次に溶出が最も早かった $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ 画分を分取し、逆相カラムに供した。

逆相カラムを用いたHPLCでは、PA化オリゴ糖をその構造によって分離することが可能である。カラムはTSKGel ODS-80T_M (4.6 x 150 mm、東ソー製) を使用し、溶媒は、100 mM酢酸アンモニウム緩衝液 (pH 4.0) (A液)、0.5% 1-ブタノールを含む100 mM酢酸アンモニウム緩衝液 (pH 4.0) (B液) を調製した。

予め溶媒Aと溶媒Bを95:5で混合したものを流速1.2 ml/minで流すことによりカラムを平衡化し、試料注入直後から溶媒Bの割合を20分かけて50%まで直線的に上昇させ、PA化オリゴ糖を溶出した。その結果を図8に示す。分取した糖鎖画分は、逆相カラムでは主に1つのピークであり (図8, a)、このピークは下記式 (III) :



(式中、Man はマンノース、GlcNAcはN-アセチルグルコサミンを示す。#は、GnT-I 作用部位を示す。)

で表される構造を有する $\text{Man}_5\text{GlcNAc}_2$ -PA標品(宝酒造製)の溶出位置と一致した(図8, b)。したがって、TIY19pGAMH1 株の生産するマンナンタンパク質には、混成型・複合型の前駆体である $\text{Man}_5\text{GlcNAc}_2$ 型の糖鎖が含まれていることが明らかとなった。

[実施例5] 混成型糖鎖($\text{GlcNAcMan}_5\text{GlcNAc}_2$)標品の合成

まず混成型糖鎖($\text{GlcNAcMan}_5\text{GlcNAc}_2$)の生合成の確認検討のため、細胞外でGnT-Iの酵素反応を用い目的糖鎖の合成を行なった。GnT-Iはその基質特異性が非常に厳密であり、前記式(III)で示される糖鎖構造に対しては#の位置のマンノース残基にのみ β -1,2 結合でGlcNAcを転移することが知られている。

ラットGnT-I遺伝子の酵母での発現は吉田らによって成功している(Yoshida et al., Glycobiology, 9, 53-58 (1999))。この遺伝子をマルチコピープラスミドであるpG3のGAP-DHプロモーターの下流に接続した後、SmaI - NaeIで切断し、プロモーターとそれに続くGnT-IのORF、ターミネーターを含む領域を切りだした。次にこの断片をマルチコピープラスミドであるpYO354のSmaI 部位に導入した。このプラスミドをpYOG4と命名した。このプラスミドを用い、野生型酵母YPH500株を酢酸リチウム法で形質転換した。形質転換後、SD-Trp (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、トリプトファンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。得られた形質転換体をYPH500/pYOG4とした。

これを500 mlのSD-Trp (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、トリプトファンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 溶液で液体培養し、集菌した。冷水で洗浄後、スフェロブ

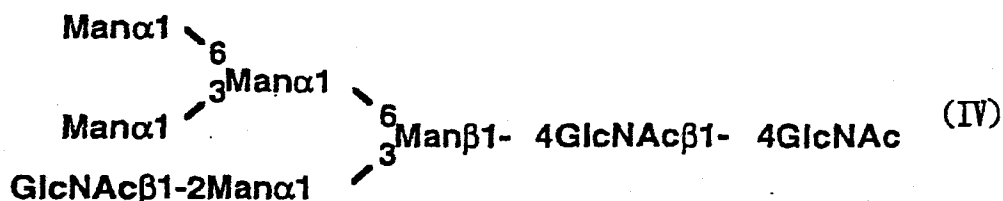
ラスト培地 (1 Mソルビトールを含む50 mM リン酸カリウム (pH 7.5)) 5.7 ml に懸濁し、2-メルカプトエタノール9 μ l と12 mgのZymolyase 100Tを300 μ l のスフェロプラスト培地に溶解し加え、30°C、45分間保温した。1 Mソルビトール15 mlを加え、遠心後、沈殿を再び1 Mソルビトール15 mlで洗浄、集菌した。この沈殿にlysis buffer (250 mMソルビトール、2 μ g/mlアンチパイン、2 μ g/mlキモスタチン、3 μ g/mlロイペプチン、3 μ g/mlペプスタチン、1 mMベンズアミジン、1 mM EDTA、1 mM EGTA、1 mM PMSFを含む10 mM トリエタノールアミン (pH 7.2)溶液) を4 ml加え、ホモジナイザーで細胞を破壊し、220 x gで遠心して上清を回収した。この上清をさらに100,000 x gで遠心し、その沈殿画分をlysis buffer 150 μ l に懸濁し、GnT-Iの酵素溶液とした。なお、本標品には他のGnT活性は検出されなかった。

次に目的糖鎖の合成を行なった。PAでラベルされたMan₅GlcNAc₂糖鎖 (宝酒造より購入) を受容体基質とし、これを200 pmol分チューブに分注した。蒸発乾固後、このチューブに上記で調製したGnT-I酵素溶液8.2 μ l、0.2 M MnCl₂ 2 μ l、GnT-I反応buffer (0.17 M MES (pH 6.0)、1.7% Triton X-100、0.34% Bovine Serum Albumin、8.47 mM AMP、1.69 mM UDP-GlcNAc、169 mM GlcNAc) 9.8 μ l を加え、37°C、3時間反応させた。5分間煮沸して反応停止後、0.22 μ m のフィルターで濾過し、HPLCに供した。

カラムはTSK Gel ODS-80T_M (4.6 x 250 mm、東ソー製) を使用し、溶媒は、0.15% 1-ブタノールを含む100 mM酢酸アンモニウム緩衝液 (pH 6.0) を用いた。予め溶媒を流速1.2 ml/minで流すことによりカラムを平衡化し、試料を注入し、PA化オリゴ糖を溶出した。その結果を図9に示す。反応物は、逆相カラムでは主に2つのピークであり、早く溶出されたピークはMan₅GlcNAc₂-PA標品 (宝酒造製、前記式(III) に構造を示す) の溶出位置と一致した。よってこれは未反応の受容体基質であると考えられた。

一方、遅く溶出されたピークについてはこれを分取し、精製の後、TOF-MSによる質量分析を行なった。ThermoQuest社製LASERMAT2000を用い、マトリックスとして2.5%の2,5-dihydroxybenzoic acid、40% アセトニトリルを含む0.01%リン酸2ナトリウムを用い、解析を行なった。その結果、前述のピーク画

分の質量は予想される分子質量 ($m/z=1521$ (H^+); $m/z=1541$ (Na^+)) とに相当した。 GnT-Iの厳密な基質特異性から、得られた糖鎖は下記式 (IV) :



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

に示す構造を有する目的の混成型糖鎖GlcNAcMan₅GlcNAc₇であると考えられた。

〔実施例 6〕 $\Delta och1 \Delta mnn1 \Delta mnn4$ 栄養要求性三重変異株への α -mannosidase I 遺伝子と GnT-I 遺伝子の導入

混成型糖鎖を酵母で生合成するには、実施例 4 で作製した酵母株にさらに GnT-I 遺伝子を導入し、発現させればよい。これにより哺乳類混成型 GlcNAcMan₅GlcNAc₂糖鎖を生合成できる。

実施例4で使用した Aspergillus saitoi 由来の α -1,2-mannosidase の小胞体型発現プラスミド pGAMH1 (Chiba et al., J. Biol. Chem., 273, 26298-26304 (1998)) より、SmaI-NaeI で切断して、プロモーターとそれに続く α -1,2-mannosidase の ORF、ターミネーターを含む領域を切りだした。次にこの断片を pYOG4 の SmaI 部位に導入した。このプラスミドを pYOMG4 と命名した。このプラスミドを用い、TIY19 株を酢酸リチウム法で形質転換した。コントロールとして、pYO354 だけで形質転換したものを用了。形質転換後、SD-Trp (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco 社製)、トリプトファンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。得られた形質転換体を TIY19 pYOMG4 とした。

この α -mannosidase I遺伝子とGnT-I遺伝子が導入され、混成型糖鎖を産生する栄養要求性三重変異株TIY19pYOMG4株は、工業技術院生命工学工業技術研究所（茨城県つくば市東1丁目1番3号）に平成11年7月2日付で受託番号

FERM BP-6775として国際寄託されている。

得られた形質転換体から実施例3と同様に糖鎖を調製、HPLCでの分析を行った。

アミドカラムによる分析結果を図10に示した。コントロールのベクターのものでは、実施例3の結果と同様、主に1つのピークであり、 $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ 標品（宝酒造製）の溶出位置と一致した（図10，A）。 α -1,2-mannosidase遺伝子とGnT-I遺伝子を含むTIY19pYOMG4では、主に5つのピークが見られた（図10，B）。これらのピークのうち、4つ（図10，B；peak a, c, d, e）は $\text{Man}_5\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_6\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_7\text{GlcNAc}_2\text{-PA}$ 、 $\text{Man}_8\text{GlcNAc}_2\text{-PA}$ 標品と溶出位置が一致した。これらはヒト型のハイマンノース型糖鎖と呼ばれるものである。さらに α -1,2-mannosidase遺伝子のみを導入したときには見られなかった新たなピーク（図10，B；peak b）が出現した。このピークの溶出位置は実施例5で作製した混成型 $\text{GlcNAcMan}_5\text{GlcNAc}_2$ 糖鎖標準品の溶出位置と一致した。さらにこのピークを分取し、実施例3と同様に逆相カラムに供した。カラム、溶媒や条件は実施例5に示した方法で行なった。分取した糖鎖画分は、逆相カラムでは主に1つのピークであり（図11）、このピークは再び $\text{GlcNAcMan}_5\text{GlcNAc}_2\text{-PA}$ 標品の溶出位置と一致した。したがって、TIY19pYOMG4株の生産するマンナンタンパク質には、混成型である $\text{GlcNAcMan}_5\text{GlcNAc}_2$ 型の糖鎖が含まれていることが明らかとなった。

〔実施例7〕 ヒト肝臓 α -mannosidase IIの酵母での発現

α -mannosidase IIは、ゴルジ体内で混成型糖鎖を一本鎖複合型糖鎖に変換する酵素である。

ヒト肝臓 α -mannosidase II遺伝子配列は、GenBankデータベースにU31520で登録されている（Misago et al., Proc. Natl. Acad. Sci., 92, 11766-11770 (1995)）。Clontech社のHuman Liver Marathon-Ready cDNAをテンプレートにして、 α -mannosidase IIのN末端側領域をコードする部分をプライマーM (CGCCGCCGAGCTCTAAAAAATGAAGTTAAGCCGCC：配列番号13)とN (ATCCCACCACTTTGAAAGGT：配列番号14)を用いて、中央をコードする部

分をプライマーO (GAAGACTCACGGAGGAAGTT : 配列番号 15) と P (ATGGCGGTATATGTGCTCGA : 配列番号 16) を用いて、C末端側領域をコードする部分をプライマーQ (CGCAGTTTGGGATACAGCAA : 配列番号 17) とプライマーR (ATTATTATTAGCGGCCGCCCTCAACTGGATTCG : 配列番号 18) を用いて、それぞれPCRで増幅した。得られたDNA断片をpCRScriptのSrfI部位に導入し、配列を確認後、BglII部位でつなぎ換えてすべての領域をコードする正しい配列になるようにした。このプラスミドをpCRMAN2と命名した。

目的のタンパク質の発現確認のため、 α -mannosidase II遺伝子の3'末端に、インフルエンザウイルスヘマグルチニンエピトープをコードする30 bpからなるHA tagを3回反復するように結合させる遺伝子を作製し、ベクターを構築した。すなわち、配列S (配列番号 19) からなる二本鎖DNAを化学合成し、発現用プラスミドのpYEX-BXのBamHIとEcoRI部位の間に挿入した。このプラスミドをpYEX-BX-3HAと命名した。次にpCRMAN2からBamHIとEcoRIで α -mannosidase IIをコードする部分を切り出し、pYEX-BX-3HAのSacIとNotI部位の間に挿入した。このプラスミドをpYEMAN2-HAと命名した。

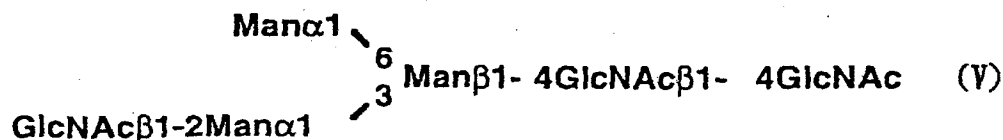
次に酵母での発現量を向上させるため、 α -mannosidase IIの膜貫通領域をコードする部分を酵母の α -1,6-mannosyltransferaseをコードする遺伝子(OCH1)の膜貫通領域部分と置換した。すなわち、配列T (配列番号 20) からなる二本鎖DNAを化学合成し、pBluescriptのSacIとEcoRI部位間に挿入した。このプラスミドをpBOCH1と命名した。一方、pYMAN2-HAをテンプレートとして、 α -mannosidase IIの触媒領域をコードする部分の一部をプライマーU (TTAGACTACCCATGGAACCCGCGCCGCGAGGGCTCCTTC : 配列番号 21) とプライマーV (CAGGAGAACTTTGGTTTCGAAAAAGCTTTGACTTCTT : 配列番号 22) を用いて増幅した。この配列を確認後、NcoIとHindIIIで切断し、pBOCH1のNcoIとHindIII部位間に挿入した。次にこのプラスミドからSacIとPstIで断片を切り出し、pYEMAN2-HAのSacIとPstI間と置換した。このプラスミドをpYEOM2-HAと命名した。

宿主として*S. cerevisiae*野生型酵母YPH500を用い、形質転換は酢酸リチウム法を用いて行なった。コントロールとしてpYEX-BX-3HAを用いた。形質転換

後、SD-Ura (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ウラシルを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。

形質転換された酵母は30℃でOD660=0.8までSD-Ura培地で培養後、硫酸銅を適量添加し、さらに2時間培養した。集菌後、SDSサンプルバッファー中でガラスビーズにより細胞を破碎し、その細胞抽出液を用いてウエスタンブロット解析を行なった。ウエスタンブロット解析は一次抗体としてラット抗HA抗体を、二次抗体として抗ラットIgG抗体ペルオキシダーゼ複合体を用い、検出はSuper Signal Ultraを基質としてX線フィルムに露光することで行なった。その結果、コントロールではシグナルが全く見られないのに対し、pYEOM2-HAで形質転換した細胞抽出液では、分子量約140000の位置にシグナルが確認された (図12)。

次に実施例5で作製した混成型糖鎖 (式(IV)に構造を示す) を基質にして、 α -mannosidase IIの酵素活性を測定した。混成型糖鎖 (式(IV)に構造を示す) 100pmolをサンプルチューブ内で乾燥させた後、同チューブに0.2 M MnCl_2 、1 M GlcNAc、1 M 酢酸ナトリウム緩衝液 (pH 5.6) を2 μl ずつ、 H_2O を8 μl 加えた後、細胞抽出液を8 μl 加えて酵素反応を開始した。37℃で一晩保温後、煮沸して反応を停止し、遠心により不溶性画分を除いたのち、HPLCにて解析した。なおHPLC分析の条件は、実施例5に従った。その結果、 α -mannosidase IIを発現させた酵母の細胞抽出液を酵素源とした場合、コントロールと比較し40分のピークが明らかに増加していた (図13、B)。この40分のピークは、PA-糖鎖標準品 (宝酒造 PA-Sugar Chain 022) の酵素消化物から得られた下記式 (V) で表される一本鎖複合型糖鎖 (Oguri et al., J. Biol. Chem., 272, 22721-22727 (1997)) の溶出位置と一致していたことから、 α -mannosidase IIの活性であることを確認した。



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

〔実施例 8〕 二本鎖複合型糖鎖を生産するのに必要な遺伝子を導入した栄養要求性三重変異株の作製

ヒト GnT-II の酵母での発現は吉田らによって報告されている (Yoshida S. et al., Abstracts of the meeting on Yeast Cell Biology, p. 279, Cold Spring Harbor Laboratory (1997))。この発現用ベクター pSY114-GnT-II よりプロモーターを含む GnT-II 遺伝子領域を XbaI で切りだし、pBluescript SK の XbaI 部位に挿入した。このプラスミドを pBlueGT2 と命名した。次に実施例 6 で示したプラスミド pYOG4 よりプロモーターを含む GnT-I 遺伝子領域を BamHI, XbaI で切り出し、pBlueGT2 の BamHI, XbaI 部位に挿入した。このプラスミドから BssHII で目的断片を切り出した後、DNA T4 polymerase で末端を平滑化した後、ADE2 をマーカーとして有する pASZ10 プラスミド (Stotz & Linder, Gene, 95, 91-98 (1990)) の SmaI 部位に断片を挿入した。このプラスミドを pASZGN12 と命名した。pASZGN12 を HpaI で直鎖状にし、実施例 1 で作製した栄養要求性三重変異株 TIY19 株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KCl を含む SD-Ade (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco 社製)、アデニンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃ で 2 日間培養し、形質転換体を得た。形質転換体よりゲノム DNA を調製し、PCR 法により GnT-I および GnT-II 遺伝子が ADE2 領域の染色体に組込まれていることを確認し、YCY22 株とした。YCY22 株の細胞抽出液を用いてそれぞれの酵素活性を測定し、GnT-I および GnT-II の発現を確認した。

一方、ヒト β -1,4-GalT の酵母での発現は、前述の吉田らによって報告されている (Yoshida S. et al., Abstracts of the meeting on Yeast Cell Biology, p. 279, Cold Spring Harbor Laboratory (1997))。この発現用ベクター pGalT13C よりプロモーターを含む β -1,4-GalT 遺伝子領域を Sall, XhoI で切りだし、pRS403 の Sall, XhoI 部位に挿入した。このプラスミドを pRSGAL1 と命名した。またヒト UDP-Gal Transporter (Ugt2p) の酵母での発現は貝沼らによって報告されてい

る (Kainuma et al., Glycobiology, 9, 133-141 (1999))。この遺伝子 (UGT2) の発現用プラスミド YEp352-GAP-UGT2 より、プロモーターを含む遺伝子領域を BamHI で切り出し、pRSGAL1 の BamHI 部位に挿入した。このプラスミドを pRSGATP1 と命名した。pRSGATP1 を NdeI で直鎖状にし、YCY22 株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KCl を含む SD-His (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco 社製)、ヒスチジンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃ で 2 日間培養し、形質転換体を得た。形質転換体よりゲノム DNA を調製し、PCR 法により β -1,4-GalT および UGT2 遺伝子が HIS3 領域の染色体に組込まれていることを確認し、YCY42 株とした。YCY42 株の細胞抽出液を用いてそれぞれの酵素活性を測定し、 β -1,4-GalT および Ugt2p の発現を確認した。

次に、ヒト肝臓 α -mannosidase II の発現用ベクター pYEOM2-HA より、SacI, SphI で HA-tag を含む遺伝子断片を切り出し、DNA T4 polymerase で末端を平滑化した。この断片を pAUR123 の SmaI 部位に挿入した。プロモーターに正しい方向でつながれていることを確認後、プロモーターを含む α -mannosidase II 遺伝子領域を BamHI で切り出し、pRS406 の BamHI 部位に挿入した。このプラスミドを NdeI で直鎖状にし、前記 YCY42 株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3M KCl を含む SD-Ura (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco 社製)、ウラシルを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃ で 2 日間培養し、形質転換体を得た。形質転換体よりゲノム DNA を調製し、PCR 法により遺伝子が URA3 領域の染色体に組込まれていることを確認し、YCY52 株とした。YCY52 株の細胞抽出液を用いて酵素活性を測定し、 α -mannosidase II の発現を確認した。

実施例 4 で示した α -1,2-mannosidase の発現用ベクター pGAMH1 より NaeI と SmaI で、プロモーター領域を含む α -1,2-mannosidase 遺伝子断片を切り出し、pYO325 ベクターの SmaI 部位に挿入した。このプラスミドを pYOM5 と命名した。さらに、ゴルジ体へ基質を供給するのに必要な UDP-GlcNAc Transporter 遺伝子

の導入を行なった。ヒトUDP-GlcNAc Transporter遺伝子の酵母での発現は石田らによって報告されている (Ishida et al., J. Biochem., 1261, 68-77 (1999))。この発現用ベクターをテンプレートにして、プライマーW (AGAGCGGCCGCAAAATGTTTCGCCAACCTAA : 配列番号23)とプライマーX (TTTTGTCTGACTAGACGCGTGAAGCATGCCC : 配列番号24)でPCR法によりUDP-GlcNAc Transporter遺伝子領域を増幅した。この配列を確認後、NotIとSalIで切断し、pG3-NのNotIとSalI部位間と置換した。次にこのプラスミドからNaeIとSmaIで、プロモーター領域を含むUDP-GlcNAc Transporter遺伝子断片を切り出し、pYOM5のSmaI部位に挿入した。このプラスミドをpYOMR5と命名した。このプラスミドを用いて前記YCY52株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-Leu (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ロイシンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。この形質転換体をYCY73株とした。YCY73株の細胞抽出液を用いて酵素活性を測定し、 α -1,2-mannosidaseとUDP-GlcNAc Transporterの発現を確認した。

また、msdSとhUGTrel2をインテグレーションするためのプラスミドを作製した。PGAMHをSmaI、NaeIで切断してGAPプロモーターとmsdS配列を切り出し、pRS404のPvu II部位に挿入した。このプラスミドをmsdS-pRS404と命名した。hUGTrel2がGAPプロモーターの下流に挿入されているプラスミド、hUGTrel2-pG3をSmaI、NaeIで切断してGAPプロモーターとhUGTrel2配列を切り出し、msdS-pRS404のPst I部位に挿入した。このプラスミドをHM-pRS404と命名した。HM-pRS404のTRP1内のBstX Iで切断し、YCY42株を酢酸リチウム法を用いて形質転換した。形質転換体を5mlのYPAD+0.3M KClで、30℃で2日間培養し、PCR法により、msdSとhUGTrel2がTRP1の染色体に組み込まれていることを確認した。また細胞抽出液を用いて酵素活性を測定し、両株の α -1,2-mannosidase及びUDP-GlcNAcトランスポーターの発現を確認した。YCY42株にmsdSとhUGTrel2をインテグレーションした株をTIY63株とした。

さらに、ヒト肝臓 α -mannosidase IIの発現用ベクターpYEOM2-HAより、

SacI, SphIでHA-tagを含む遺伝子断片を切り出し、DNA T4 polymeraseで末端を平滑化した。この断片をpAUR123のSmaI部位に挿入した。プロモーターに正しい方向でつながれていることを確認後、プロモーターを含む α -mannosidase II遺伝子領域をBamHIで切り出し、pRS406のBamHI部位に挿入した。このプラスミドをNdeIで直鎖状にし、TIY63株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-Ura (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ウラシルを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30°Cで2日間培養し、形質転換体を得た。形質転換体よりゲノムDNAを調製し、PCR法により遺伝子がURA3領域の染色体に組込まれていることを確認し、MSY3株とした。MSY3株の細胞抽出液を用いて酵素活性を測定し、 α -mannosidase IIの発現を確認した。

〔実施例9〕 二本鎖複合型糖鎖を生産するのに必要な遺伝子を導入した栄養要求性四重変異株の作製

まず、実施例8で作製したプラスミドpASZGN12をHpaIで直鎖状にし、実施例2で作製した栄養要求性四重変異株YS134-4A株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-Ade (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、アデニンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30°Cで2日間培養し、形質転換体を得た。形質転換体よりゲノムDNAを調製し、PCR法によりGnT-IおよびGnT-II遺伝子がADE2領域の染色体に組込まれていることを確認し、YCY122株とした。YCY122株の細胞抽出液を用いてそれぞれの酵素活性を測定し、GnT-IおよびGnT-IIの発現を確認した。

次に、実施例8で作製したプラスミドpRSGATP1をNdeIで直鎖状にし、YCY122株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-His (2%グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ヒスチジンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30°Cで2日間培養し、形質転換体を得た。形

質転換体よりゲノムDNAを調製し、PCR法により β -1,4-GalTおよびUGT2遺伝子がHIS3領域の染色体に組込まれていることを確認し、YCY142株とした。YCY142株の細胞抽出液を用いてそれぞれの酵素活性を測定し、 β -1,4-GalTおよびUgt2pの発現を確認した。

さらに、実施例8で示したプラスミドpYOMR5を用いてYCY142株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-Leu (2% グコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ロイシンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。この形質転換体をYCY163株とした。YCY163株の細胞抽出液を用いて酵素活性を測定し、 α -1,2-mannosidaseとUDP-GlcNAc Transporterの発現を確認した。

このYCY163株について、酵母の細胞表層のマナンタンパク質の糖鎖構造の変化を見るために、レクチン染色性の評価を行なった。コンカナバリンAは特定のマンノース3残基を含む、ハイマンノース型、混成型、二本鎖複合型糖鎖などと結合することが知られているが、その親和性は混成型、二本鎖複合型糖鎖と比べハイマンノース型糖鎖の方が高いことが知られている。そこで、Texas-red標識コンカナバリンA溶液を集菌した酵母細胞と混合し、時々攪拌しながら4℃、2時間放置した。PBSで洗浄後、10 mM α -methyl mannosideを含むPBSで洗浄し、蛍光顕微鏡下で観察を行なった。その結果、YS134-4A株では洗浄後も細胞の周りが蛍光染色されていたが、YCY163株では細胞の周囲にみられた蛍光が明らかに減少していることを確認した。このことからYCY163株ではハイマンノース型の糖鎖が減少し、複合型糖鎖が生成していることが示唆された。

〔実施例10〕 哺乳類型糖鎖生産能を有する酵母変異株でのヒト線維芽細胞成長因子 (FGF) の生産と糖鎖構造の改変

FGF6-1キメラ遺伝子 (secFGF (N35)) は生命工学工業技術研究所の米田敦子氏より供与頂いた (Yoneda et al., BioTechniques, 27, 576-590 (1999))。SecFGF(N35)/pBSをSma I、Nae Iで切断してFGFを切り出し、pGEM2- α 36のHindIII部位に挿入した。このプラスミドをpFGF α 23と命名した。pFGF α 23

をEcoR Iで切断し、prepro α -factorとFGF領域を切り出し、pUC119プラスミドのEcoR I部位に挿入した。このプラスミドをFGF-pUC119と命名した。 α -factor の EAEA 配列を除くために、プライマー Y (CGCCAGGGTTTTCCCAGTCACGAC : 配列番号 25) とプライマー Z (ATGGGCCGGCTCTTTTATCCAAAGATAC : 配列番号 26) を用いてPCRで増幅した。このDNA断片をpUC18のEcoR I部位に組み込み、pAF02プラスミドを作製した。pFGF01をNae I、Sma Iで切断してFGFを切り出し、pAF02のNae I、Sma I部位に挿入した。このプラスミドをpAF03と命名した。pAF03をEcoR Iで切断してprepro α -factorとFGF領域を切り出し、YEp352GAPプラスミドのGAPプロモーターの下流に組み込んだプラスミドpAFF2を作製した。pAFF2をAat II、Hpa Iで切断し、2 μ m領域を切り出し、酵母インテグレーション用プラスミドpAFF3を構築した。次に、pAFF3をApaI I、Acc Iで切断してGAPプロモーターとFGFの配列を切り出し、LEU2マーカーをもつプラスミドpYO325のPvu II部位に挿入した。さらにプラスミドの2 μ m領域をSpe Iで切断して除いた。このプラスミドをpAFF9と命名した。pAFF9内にあるEcoR Vで切断して直鎖化し、酵母 (TIY19株、YCY42株) を酢酸リチウム法を用いて形質転換した。形質転換後、SD-Leu (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ロイシンを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L)) 培地のプレートにまいて、30°Cで2日間培養し、それぞれ形質転換体を得た。

それらの形質転換体を5mlのYPAD+0.3M KClで、30°Cで3日間培養し、培養上清液に50 μ lベッドのヘパリンセファロース懸濁液 (ファルマシア社製) を加え、4°Cで一晩振とうしてFGFをヘパリンセファロースに吸着させた。その後、遠心にてヘパリンセファロースを回収し、SDSサンプルバッファーで煮沸後、上清をSDS-PAGEに供した。抗FGF抗体を用いてウエスタンブロッティングを行い、FGFが発現していることを確認した。さらに、PCR法により、FGFがLEU2の染色体に組み込まれていることを確認し、TIY19株にFGFをインテグレーションした株をTIY48株、YCY42株にFGFをインテグレーションした株をTIY49株とした。

さらにタンパク質の安定的かつ効率的な発現を行なうため、msdSをインテグレーションするためのプラスミドを作製した。msdSがGAPプロモーターの下流に挿入されているプラスミドpGAMHをEcoR Iで切断し、2 μ m領域を除いたプラスミドを作製した。このプラスミドをpImsdSと命名した。pImsdSのTRP1内のXba Iで切断し、TIY48 (Δ mnn1::hisG Δ mnn4::hisG Δ och1::hisG FGF::LEU2)、TIY49 (Δ mnn1::hisG Δ mnn4::hisG Δ och1::hisG FGF::LEU2 ade2::[GnT-I & GnT-II] his3::[β -1.4-GalT & UGT2])を酢酸リチウム法を用いて形質転換した。得られた形質転換体を5 mlのYPAD+0.3 M KClで、30℃で3日間培養し、培養液に50 μ lのヘパリンセファロース（ファルマシア製）を加え、4℃で一晩振とうしてFGFをヘパリンセファロースに吸着させた。その後、ヘパリンセファロースを回収し、FGFの抗体でウエスタンブロッティングを行い、msdSが発現していることを確認した。さらに、PCR法により、msdSがTRP1の染色体に組み込まれていることを確認した。TIY48株にmsdSをインテグレーションした株をTIY53株、TIY49株にmsdSをインテグレーションした株をTIY54株とした。

次に、msdSとhUGTrel2をインテグレーションするためのプラスミドを作製した。PGAMHをSma I、Nae Iで切断してGAPプロモーターとmsdS配列を切り出し、pRS404のPvu II部位に挿入した。このプラスミドをmsdS-pRS404と命名した。hUGTrel2がGAPプロモーターの下流に挿入されているプラスミド、hUGTrel2-pG3をSma I、Nae Iで切断してGAPプロモーターとhUGTrel2配列を切り出し、msdS-pRS404のPst I部位に挿入した。このプラスミドをHM-pRS404と命名した。HM-pRS404のTRP1内のBstX Iで切断し、TIY48株、TIY49株を酢酸リチウム法を用いて形質転換した。形質転換体を5mlのYPAD+0.3M KClで、30℃で3日間培養し、培養上清液に50 μ lベッドのヘパリンセファロース懸濁液（ファルマシア製）を加え、4℃で一晩振とうしてFGFをヘパリンセファロースに吸着させた。その後、遠心にてヘパリンセファロースを回収し、SDSサンプルバッファーで煮沸後、上清をSDS-PAGEに供した。抗FGF抗体を用いてウエスタンブロッティングを行い、FGFが発現していることを確認した。さらに、PCR法により、msdSとhUGTrel2がTRP1の染色体に組み

込まれていることを確認した。また細胞抽出液を用いて酵素活性を測定し、両株の α -1,2-mannosidase及びUDP-GlcNAcトランスポーターの発現を確認した。TIY48株にmsdSとhUGTrel2をインテグレーションした株をTIY59株、TIY49株にmsdSとhUGTrel2をインテグレーションした株をTIY60株とした。

糖鎖の調製のため、FGFをLEU2の染色体上にインテグレーションしたTIY48株とTIY53株を使用して、3 Lの培養液からFGFを精製した。3 LのYPAD+0.3 M KClで30℃、3日間培養後、遠心して細胞を除いた培養液に2 mlのヘパリンセファロースを加え、4℃で一晩振とうしてFGFをヘパリンセファロースに吸着させた。ヘパリンセファロースを回収し、カラムにつめ、PBS+0.01% CHAPS、PBS+2.5 M NaCl+0.01% CHAPSを溶媒として、塩濃度の上昇によりFGFをヘパリンセファロースから溶出させた。

精製したFGF約150 μ gを逆相カラムにかけることにより、脱塩した。カラムは μ RPC C2/C18 PC3.2/3カラム（ファルマシア社製）を使用し0.1% トリフルオロ酢酸と0.1% トリフルオロ酢酸 - 60% アセトニトリルを溶媒として、逆相カラムからの溶出を行なった。

カラムから溶出したサンプルを乾燥させてヒドラジン分解を行った。真空状態で2 mlのヒドラジンを加え、110℃で60分間処理した。その後、室温まで冷却し、N-アセチル化を行った。250 μ lの0.2M酢酸アンモニウムと25 μ lの無水酢酸を加えよく攪拌し、30分間室温で放置した。さらに、250 μ lの0.2M酢酸アンモニウムと25 μ lの無水酢酸を加えよく攪拌し、30分間室温で放置した。反応液を濃縮乾固し、糖鎖調整品とした。

得られた糖鎖を蛍光標識（ピリジルアミノ化）するため、以下の操作を行った。糖鎖調整品を濃縮乾固後、20 μ lのカップリング試薬（300 mgの2-アミノピリジンを100 μ lの酢酸に溶解した）を加え、密封し、90℃、60分間処理した。その後、20 μ lの還元試薬（10 mgのボラン・ジメチルアミン複合体を50 μ lの酢酸に溶解した）を加え、密封し、80℃、60分間処理した。反応後、20 μ lのトリエチルアミン-メタノールを添加しよく攪拌した後、さらに40 μ lのトルエンを加えよく攪拌して、60℃、10分間、窒素気流下濃縮乾固した。その後、反応液に、20 μ lのメタノールを加えよく攪拌した後、40 μ lのトルエンを加えよく

攪拌して、60℃、10分間、窒素気流下濃縮乾固した。これを三回繰り返して、残渣に50 μ lのトルエンを加え、60℃、10分間、窒素気流下濃縮乾固した。反応後、HW-40ゲル濾過カラム処理を行い、未反応の2-アミノピリジンを除去した。

アミノカラムを用いたHPLCにより糖鎖構造解析を行った。カラムはAsahipak NH2P-50 (4.6 mm \times 250 mm) を使用し、溶媒は、200 mM酢酸-トリエチルアミン緩衝液 (pH 7.3) とアセトニトリルとの7:3の混合液 (A液)、200 mM酢酸-トリエチルアミン緩衝液 (pH 7.3) とアセトニトリルとの2:8の混合液 (B液) を調整した。

予め溶媒Aを流速1.0 ml/minで流すことによりカラムを平衡化し、試料注入直後から溶媒Bの割合を50分かけて100%まで直線的に上昇させ、その後、溶媒Bの割合を100%のまま20分間流し、PA化オリゴ糖を溶出した。その分析結果を図14に示した。TIY48由来のものでは、実施例2の結果と同様、主に1つのピークであり (図14、上段)、Man8GlcNAc2-PA標品 (宝酒造製) の溶出位置と一致した。一方、 α -1,2-mannosidase 遺伝子を含むTIY53株では、主に1つのピークが見られた (図14、下段)。このピークはMan5GlcNAc2-PA標品と溶出位置が一致した。したがって、TIY53株で発現させたヒト糖タンパク質であるFGFは、ほぼ100%混成型・複合型の前駆体であるMan5GlcNAc2型糖鎖を有することが明らかとなった。

さらに、ヒト肝臓 α -mannosidase IIの発現用ベクターpYEOM2-HAより、SacI, SphIでHA-tagを含む遺伝子断片を切り出し、DNA T4 polymeraseで末端を平滑化した。この断片をpAUR123のSmaI部位に挿入した。プロモーターに正しい方向でつながれていることを確認後、プロモーターを含む α -mannosidase II遺伝子領域をBamHIで切り出し、pRS406のBamHI部位に挿入した。このプラスミドをNdeIで直鎖状にし、TIY60株の形質転換を酢酸リチウム法で行なった。形質転換後、0.3 M KClを含むSD-Ura (2% グルコース、0.67 % Yeast Nitrogen Base w/o amino acids (Difco社製)、ウラシルを除く核酸塩基およびアミノ酸混合物 (20-400 mg/L) 培地のプレートにまいて、30℃で2日間培養し、形質転換体を得た。形質転換体よりゲノムDNAを調製し、PCR法により遺伝子がURA3領域の染色体に組込まれていることを確認し、MSY1株とした。MSY1

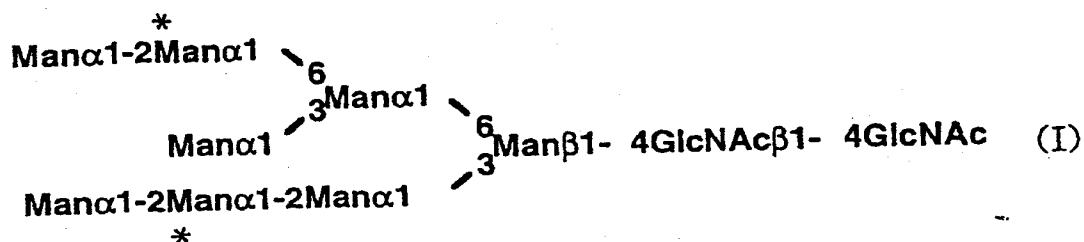
株の細胞抽出液を用いて酵素活性を測定し、 α -mannosidase IIの発現を確認した。

産業上の利用可能性

本発明により新規に育種した栄養要求性三重変異株、栄養要求性四重変異株によれば、ヒトなど哺乳類細胞の生産するハイマンノース型と同一の中性糖鎖、あるいは同一の中性糖鎖を有する糖タンパク質を多量かつ純度よく生産することができる。また、当該変異株に哺乳類型糖鎖の生合成系遺伝子を導入することにより、ハイマンノース型、ハイブリッド型、複合型等の哺乳類型糖鎖、あるいは哺乳類型糖鎖を有するタンパク質を効率的に生産することができる。

請 求 の 範 囲

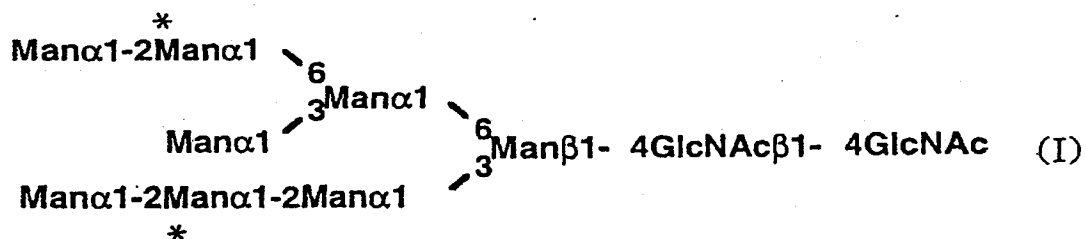
1. och1変異、mnn1変異、mnn4変異の変異形質と、少なくとも4個以上の栄養要求性変異形質を持つことを特徴とし、下記式 (I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。＊はリン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

2. 栄養要求性を相補する遺伝子を最終的に導入することなく、OCH1遺伝子を破壊したoch1変異 ($\Delta och1$)、MNN1遺伝子を破壊したmnn1変異 ($\Delta mnn1$)、MNN4遺伝子を破壊したmnn4変異 ($\Delta mnn4$) の変異形質と、少なくとも1個以上の栄養要求性変異形質を持つことを特徴とし、下記式 (I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。＊はリン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

3. 栄養要求性変異形質が、ura3変異, his3変異, leu2変異, ade2変異, trp1

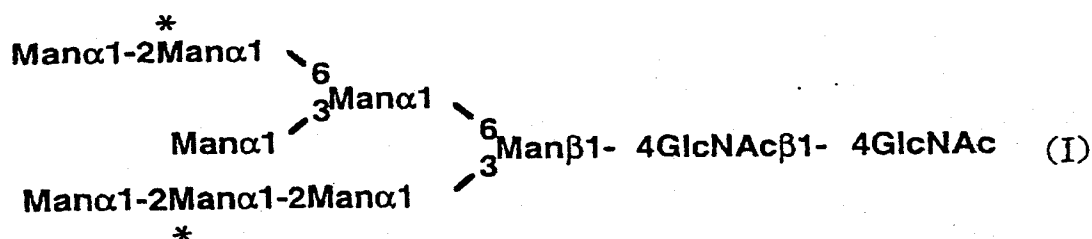
変異、can1変異から選ばれる、請求項 1 または 2 に記載の酵母変異株。

4. サッカロミセス (Saccharomyces)属に属する酵母である、請求項 3 に記載の酵母変異株。

5. サッカロミセス・セレビシエ (Saccharomyces cerevisiae) に属する酵母である、請求項 4 に記載の酵母変異株。

6. サッカロミセス・セレビシエ (Saccharomyces cerevisiae) TIY19 株である、請求項 5 に記載の酵母変異株。

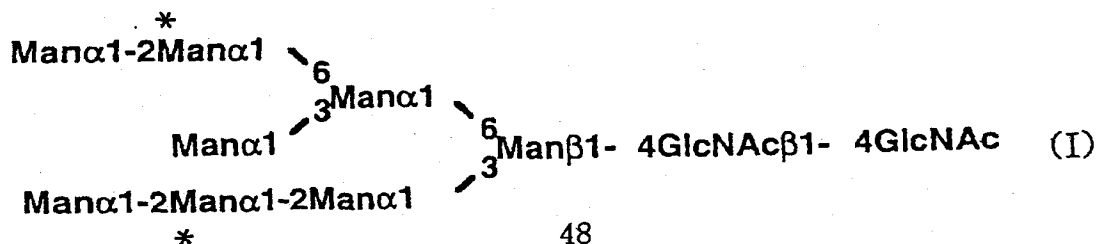
7. 請求項 1 ～ 6 のいずれか 1 項に記載の酵母変異株を培地に培養し、培養物中に下記式 (I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。*はリン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

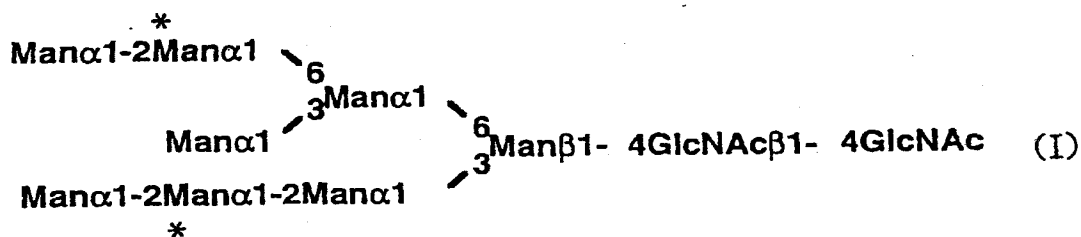
8. 請求項 1 ～ 6 のいずれか 1 項に記載の酵母変異株を培地に培養し、培養物中に下記式 (I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。＊はリン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

9. 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた請求項1～6のいずれか1項に記載の酵母変異株を培地に培養し、培養物中に下記式 (I):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。＊はリン酸化可能部位を示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

10. och1変異、mnn1変異、mnn4変異の変異形質を持つ酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも二つ以上導入した酵母変異株。

11. 請求項1～6のいずれか1項に記載の酵母変異株に、哺乳類型糖鎖の合成系遺伝子を、少なくとも一つ以上導入した酵母変異株。

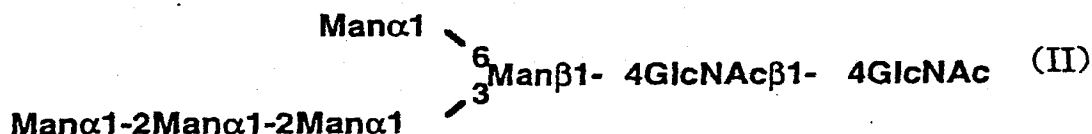
12. 請求項10または11に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖

を回収することを特徴とする、オリゴ糖鎖の製造法。

13. 請求項10または11に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

14. 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた請求項10または11に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

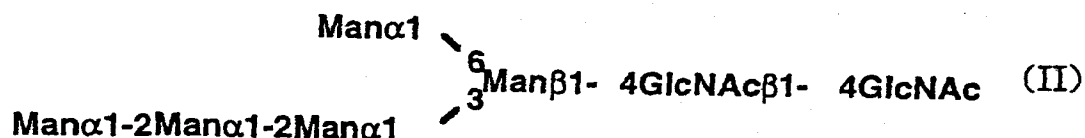
15. och1変異、mnn1変異、mnn4変異、alg3変異の変異形質と、少なくとも5個以上の栄養要求性変異形質を持つことを特徴とし、下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

16. 栄養要求性を相補する遺伝子を最終的に導入することなく、OCH1遺伝子を破壊したoch1変異 (Δoch1)、MNN1遺伝子を破壊したmnn1変異 (Δmnn1)、MNN4遺伝子を破壊したmnn4変異 (Δmnn4)、ALG3遺伝子を破壊したalg3変異 (Δalg3) の変異形質と、少なくとも1個以上の栄養要求性変異形質を持つことを特徴とし、下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質生産能を有する酵母変異株。

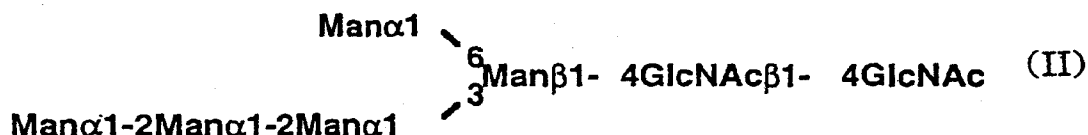
17. 栄養要求性変異形質が ura3変異, his3変異, leu2変異, ade2変異, trp1変異, can1変異から選ばれる、請求項15または16に記載の酵母変異株。

18. サッカロミセス (*Saccharomyces*)属に属する酵母である、請求項17に記載の酵母変異株。

19. サッカロミセス・セレビシエ (*Saccharomyces cerevisiae*) に属する酵母である、請求項18に記載の酵母変異株。

20. サッカロミセス・セレビシエ (*Saccharomyces cerevisiae*) YS134-4A株である、請求項19に記載の酵母変異株。

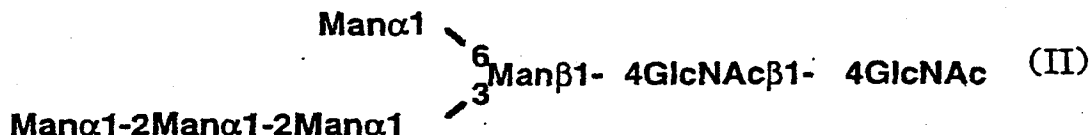
21. 請求項15～20のいずれか1項に記載の酵母変異株を培地に培養し、培養物中に下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

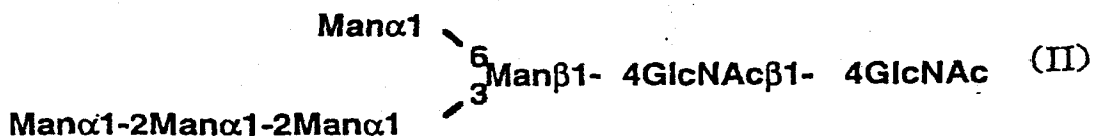
22. 請求項15～20のいずれか1項に記載の酵母変異株を培地に培養し、培養物中に下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

23. 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた請求項15～20のいずれか1項に記載の酵母変異株を培地に培養し、培養物中に下記式 (II):



(式中、Manはマンノース、GlcNAcはN-アセチルグルコサミンを示す。)

で表されるオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

24. och1変異、mnn1変異、mnn4変異、alg3変異の変異形質を持つ酵母変異株に、哺乳類型糖鎖の生合成系遺伝子を、少なくとも二つ以上導入した酵母変異株。

25. 請求項15～20のいずれか1項に記載の酵母変異株に、哺乳類型糖鎖の

生合成系遺伝子を、少なくとも一つ以上導入した酵母変異株。

26. 請求項24または25に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取し、採取した糖タンパク質より該オリゴ糖鎖を回収することを特徴とする、オリゴ糖鎖の製造法。

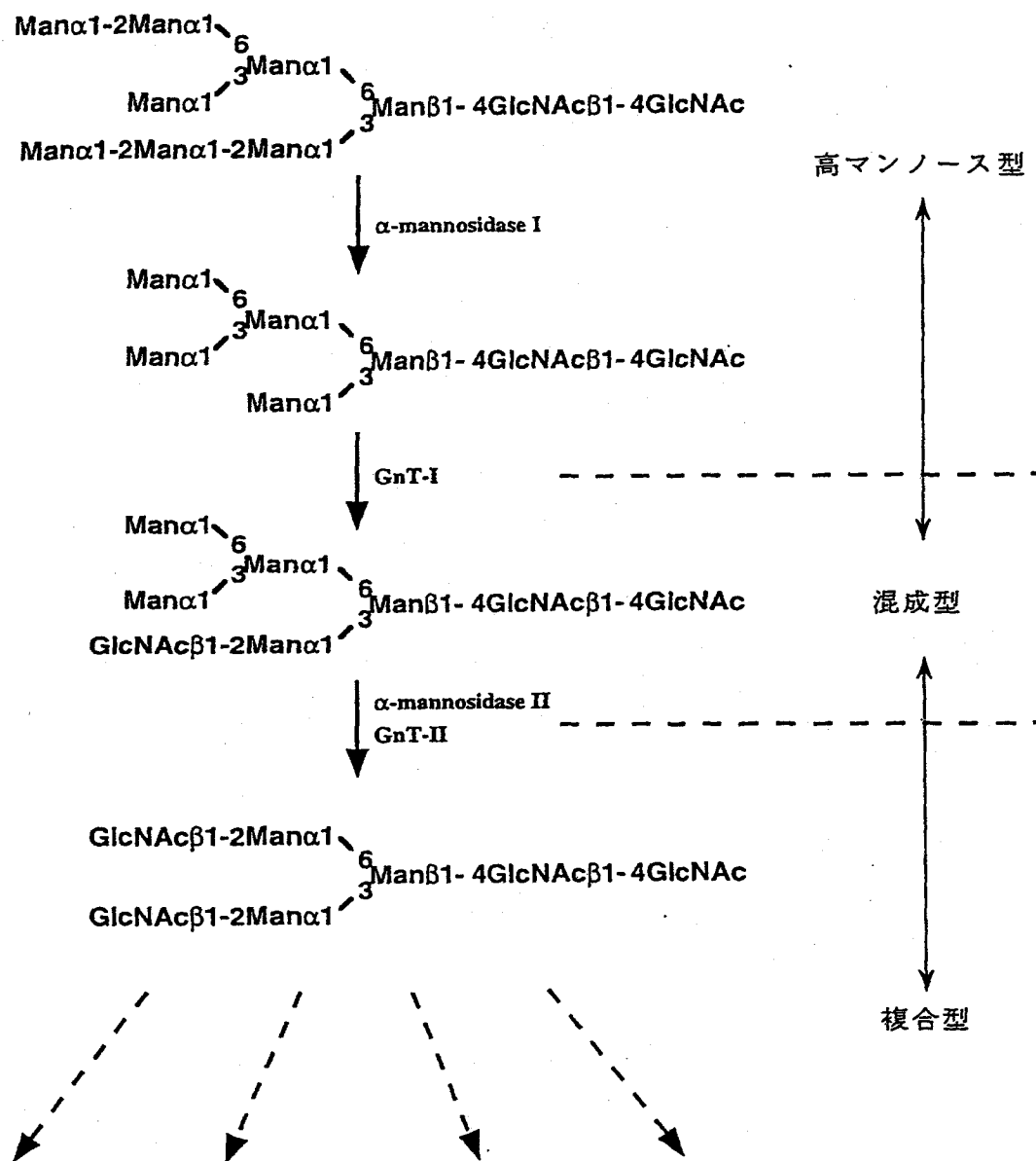
27. 請求項24または25に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

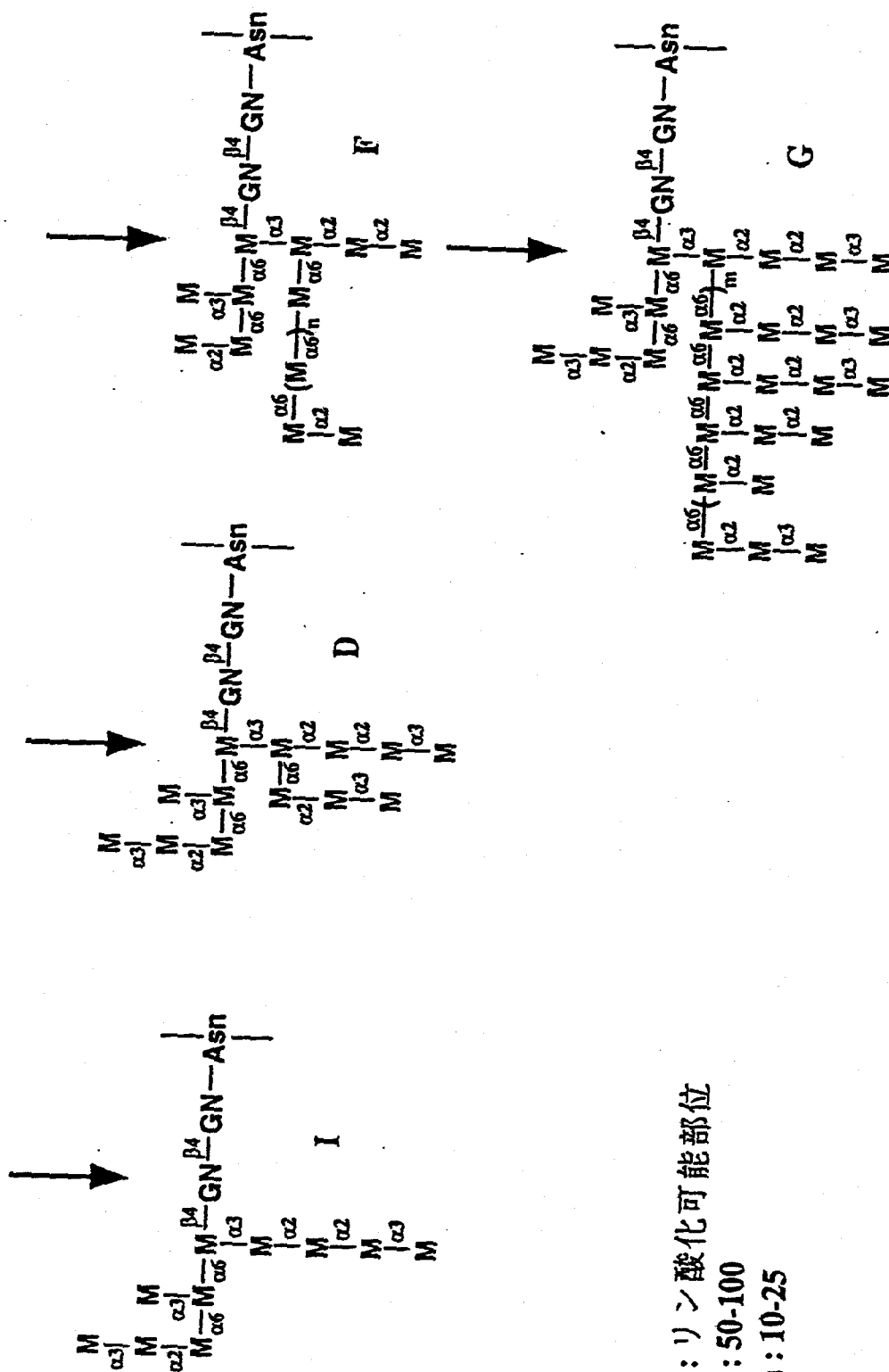
28. 哺乳類由来のアスパラギン結合型糖タンパク質をコードする遺伝子を含む組換えプラスミドにて形質転換させた請求項24または25に記載の酵母変異株を培地に培養し、培養物中にオリゴ糖鎖をアスパラギン結合型糖鎖として含有する糖タンパク質を生成蓄積させ、該培養物から該糖タンパク質を採取することを特徴とする、糖タンパク質の製造法。

29. α -マンノシダーゼII遺伝子が導入され、 α -マンノシダーゼII活性を有する酵母株。

30. 請求項29に記載の酵母株を培地に培養し、培養物中に生成蓄積された α -マンノシダーゼIIを採取することを特徴とする、 α -マンノシダーゼIIの製造法。

図 1





*: 1) 酸化可能部位

n: 50-100

m: 10-25

図 4

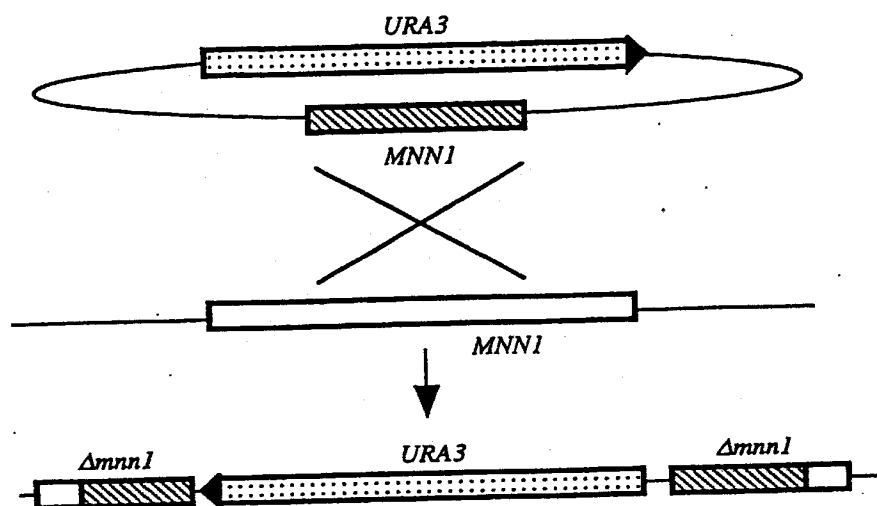


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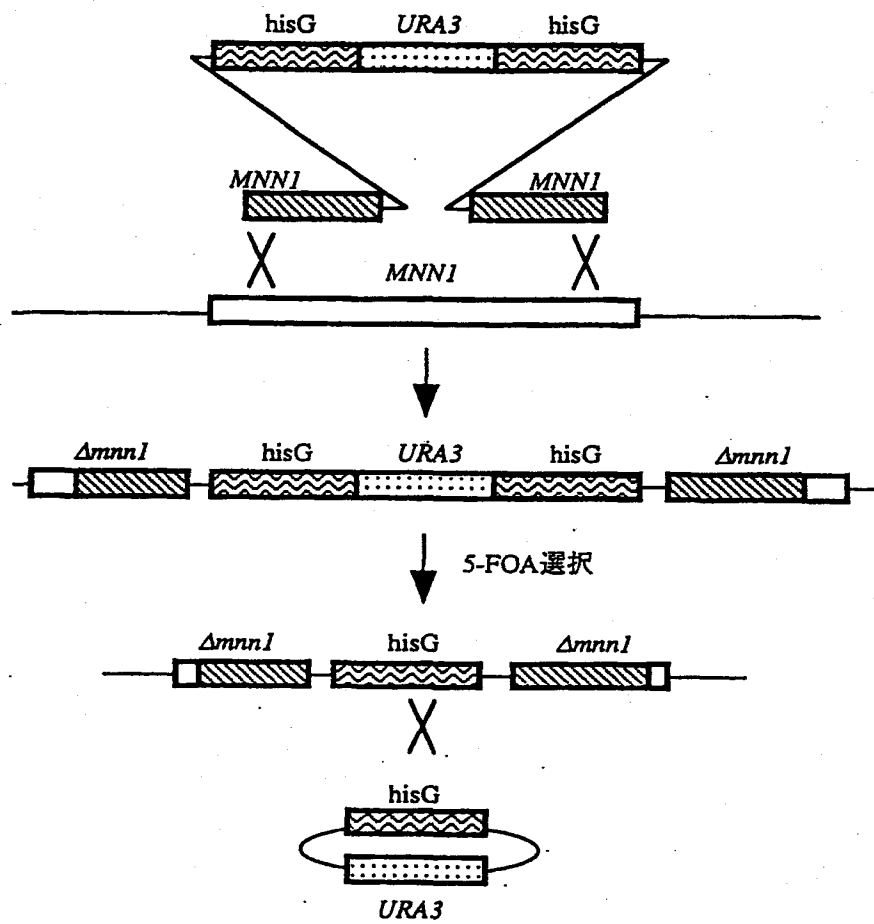


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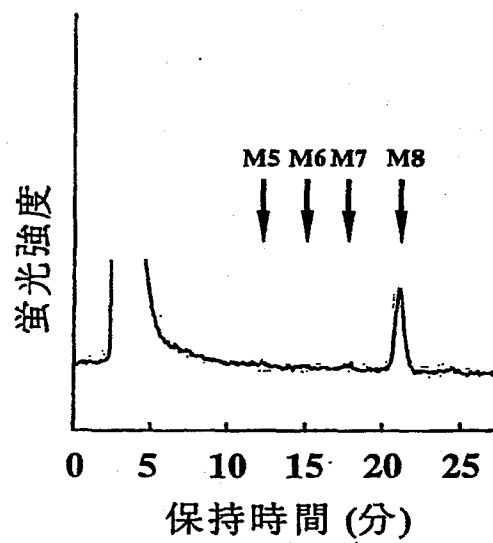


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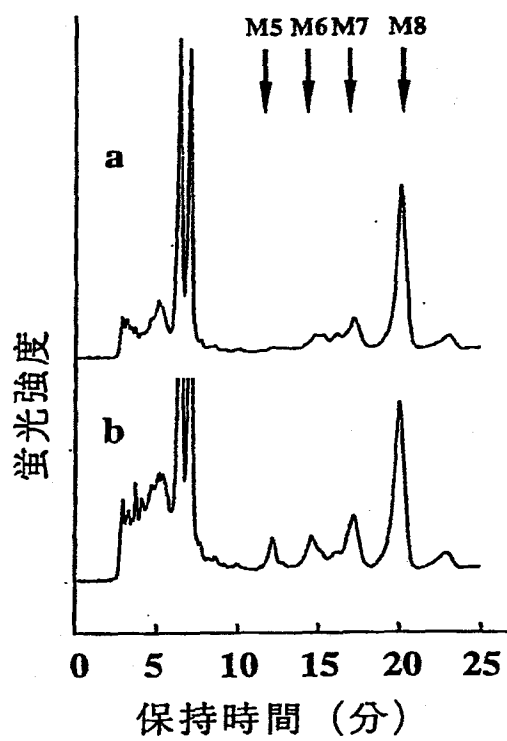


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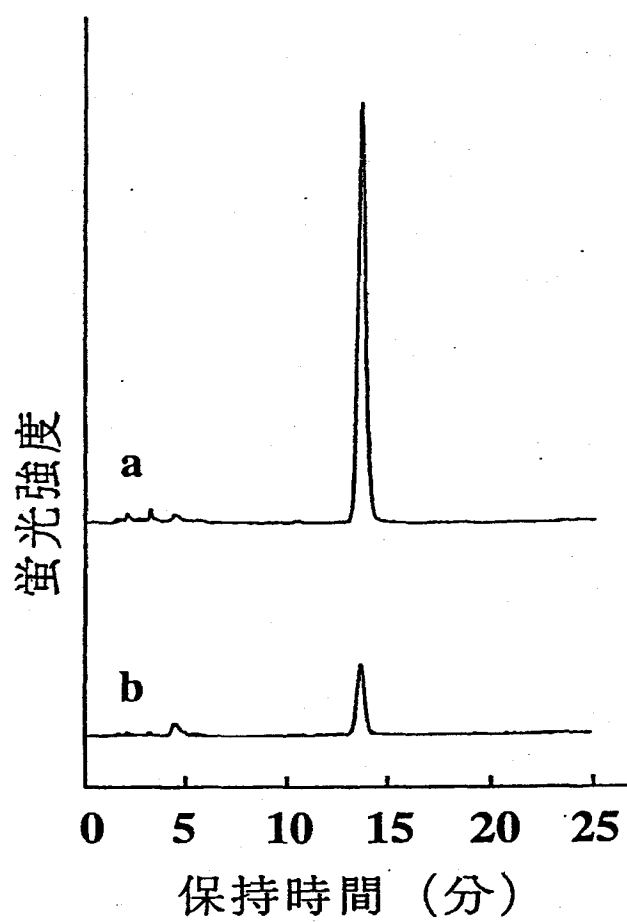


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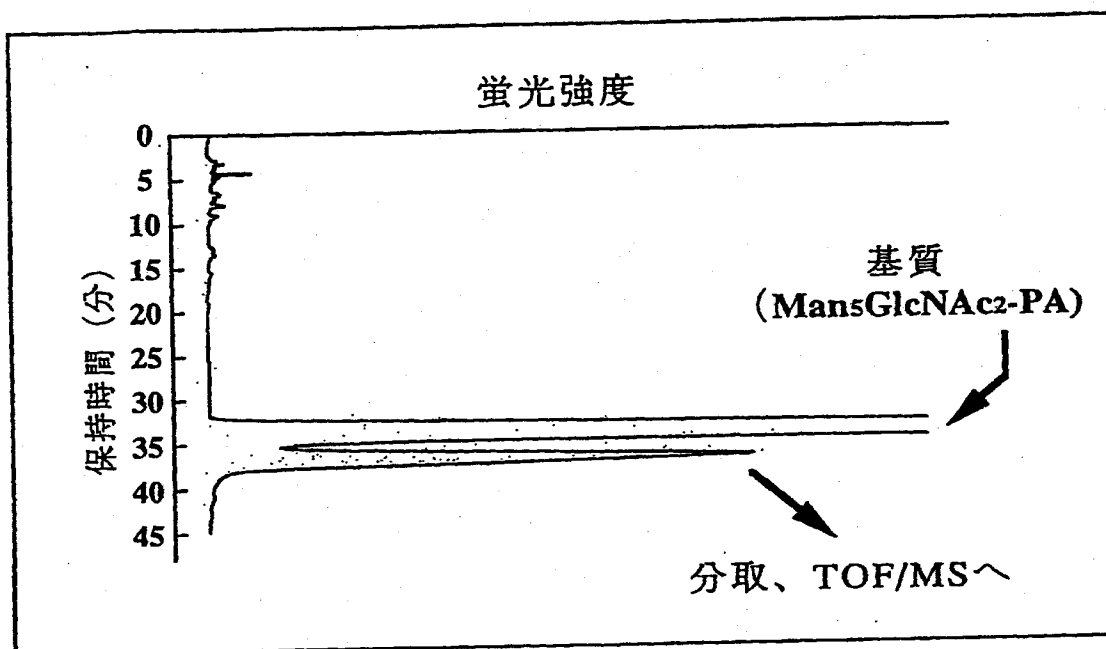


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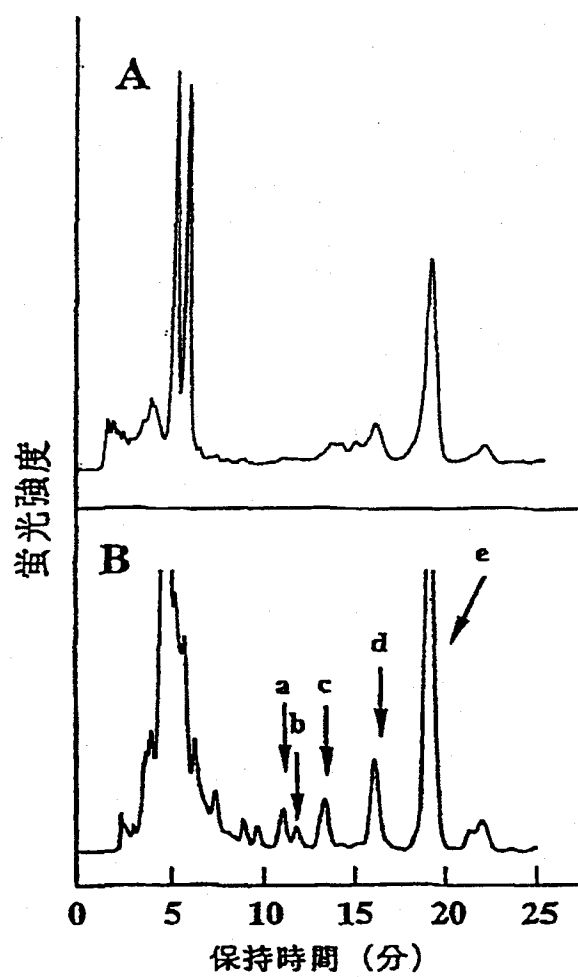
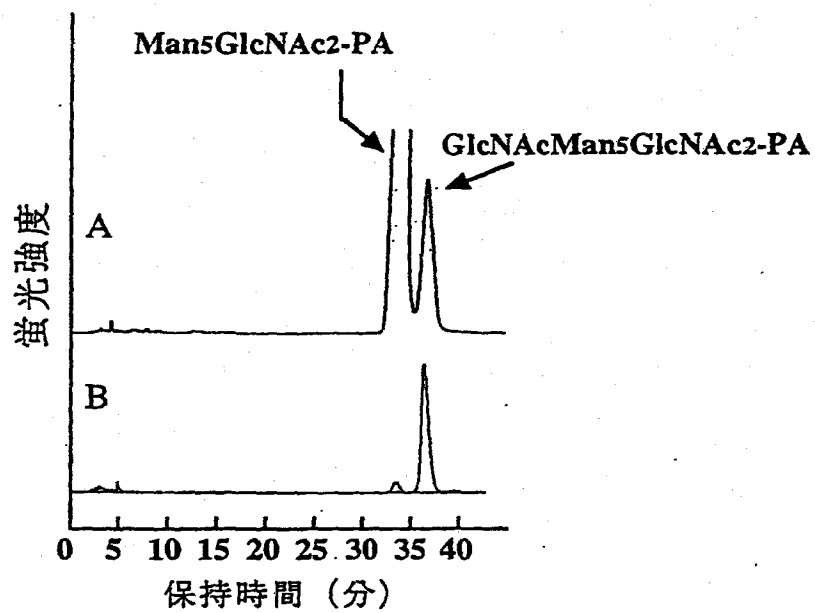


図 1 1



☒ 1 2

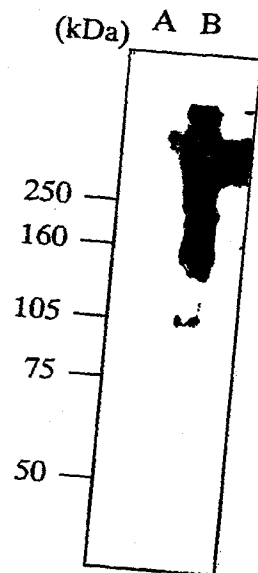


図 13

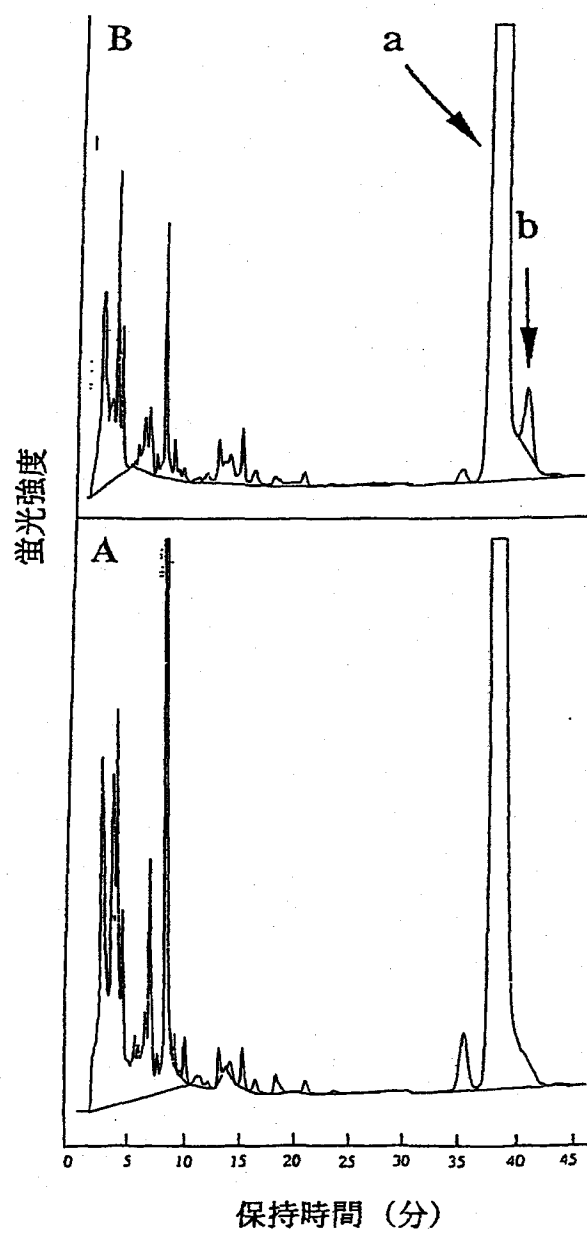
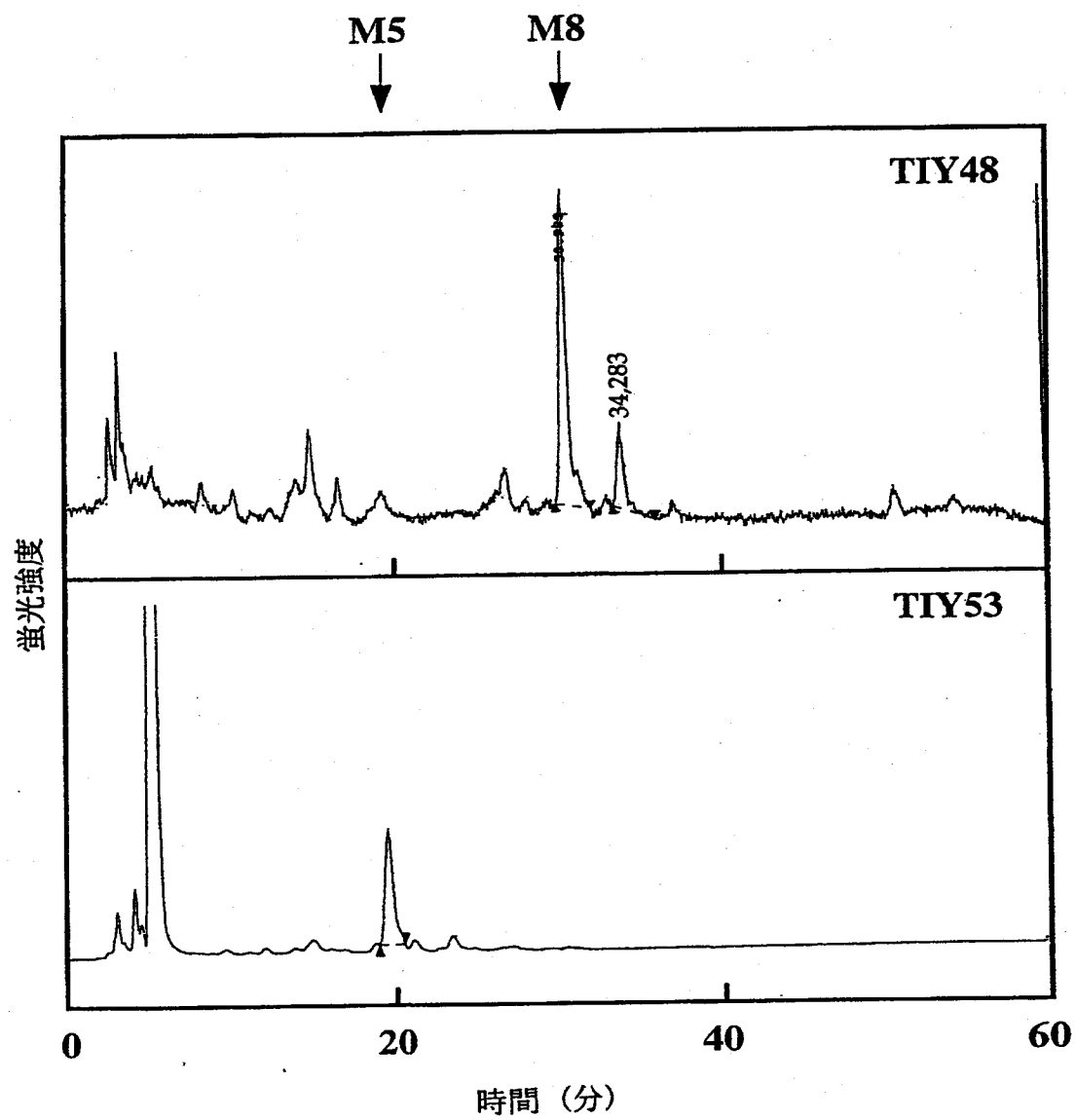


図 1 4



配列表

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Secretary of Agency of Industrial Science and Technology

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with mammalian-typed sugar chains

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<170> PatentIn Ver. 2.0

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28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/05474

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl⁷ C12N 1/19, C12P 21/02 // (C12N 1/19, C12R 1:865), (C12P 21/02, C12R 1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl⁷ C12N 1/19, C12P 21/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, WPI/L, BIOSIS PREVIEWS, CAS ONLINE, DDBJ/EMBL/GenBank/Geneseq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nakayama K. et al. "OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides" EMBO J., Vol.11 (1992) pp.2511-2519	1-30
A	Odani T. et al., "Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the MNN4 gene in Saccharomyces cerevisiae" FEBS letters, Vol.420 (1997) pp.186-190	1-30
A	Nakanishi Y. et al., "Structure of the N-Linked Oligosaccharides That Show the Complete Loss of α -1,6-Polymannose Outer Chain from och1, och1 mnn1, and och1 mnn1 alg3 Mutants of Saccharomyces cerevisiae" J. Biol. Chem., Vol.268 (1993) pp.26338-26345	1-30

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
14 November, 2000 (14.11.00)Date of mailing of the international search report
05 December, 2000 (05.12.00)Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

A. 発明の属する分野の分類 (国際特許分類 (IPC))		
Int. Cl' C12N 1/19, C12P 21/02 // (C12N 1/19, C12R 1:865), (C12P 21/02, C12R 1:865)		
B. 調査を行った分野		
調査を行った最小限資料 (国際特許分類 (IPC))		
Int. Cl' C12N 1/19, C12P 21/02		
最小限資料以外の資料で調査を行った分野に含まれるもの		
国際調査で使用した電子データベース (データベースの名称、調査に使用した用語)		
WPI, WPI/L, BIOSIS PREVIEWS, CAS ONLINE, DDBJ/EMBL/GenBank/Geneseq		
C. 関連すると認められる文献		
引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
A	Nakayama K. et al. "OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides" EMBO J., 第11巻 (1992) p. 2511-2519	1-30
A	Odani T. et al. "Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the MNN4 gene in Saccharomyces cerevisiae" FEBS letters, 第420巻 (1997) p. 186-190	1-30
<input checked="" type="checkbox"/> C欄の続きにも文献が列挙されている。 <input type="checkbox"/> パテントファミリーに関する別紙を参照。		
* 引用文献のカテゴリー 「A」 特に関連のある文献ではなく、一般的技術水準を示すもの 「E」 国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの 「L」 優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献 (理由を付す) 「O」 口頭による開示、使用、展示等に言及する文献 「P」 国際出願日前で、かつ優先権の主張の基礎となる出願日の後に公表された文献 「T」 国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの 「X」 特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの 「Y」 特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの 「&」 同一パテントファミリー文献		
国際調査を完了した日	14. 11. 00	国際調査報告の発送日
		05. 12. 00
国際調査機関の名称及びあて先	特許庁審査官 (権限のある職員)	4 B 9 0 5 0
日本国特許庁 (ISA/JP)	加藤 浩	印
郵便番号 100-8915	電話番号 03-3581-1101	内線 3448
東京都千代田区霞が関三丁目4番3号		

C (続き) . 関連すると認められる文献		
引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
A	Nakanishi Y. et al. "Structure of the N-Linked Oligosaccharides That Show the Complete Loss of α -1,6-Polymannose Outer Chain from och1, och1 mnn1, and och1 mnn1 alg3 Mutants of <i>Saccharomyces cerevisiae</i> " J. Biol. Chem., 第268巻 (1993) p. 26338-26345	1-30

WO 01/14522

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.*** shows the word which can not be translated.
3.In the drawings, any words are not translated.

[Claim(s)]

*

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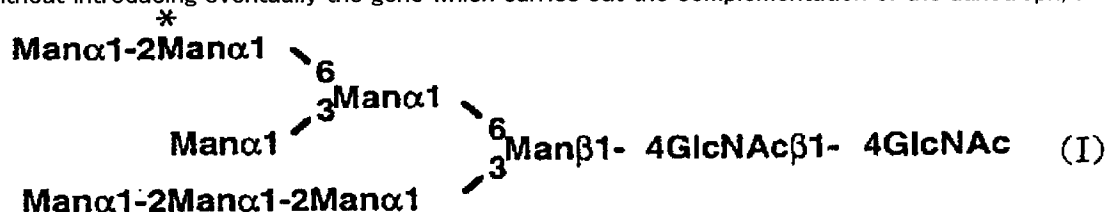
6
3
Man α 1

6
3
Man β 1- 4GlcNAc β 1- 4GlcNAc (I)

6
3
Man α 1-2Man α 1-2Man α 1

*

[Claim 2] It is characterized by having the quality of a variant of och1 variation (deltaoch1) which destroyed OCH1 gene, mnn1 variation (deltamnn1) which destroyed MNN1 gene, and mnn4 variation (deltamnn4) which destroyed MNN4 gene, and at least one or more quality of an auxotroph variant, without introducing eventually the gene which carries out the complementation of the auxotroph, and is the



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.) * A phosphorylation possible part is shown. The yeast variant which has the glycoprotein productivity which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain.

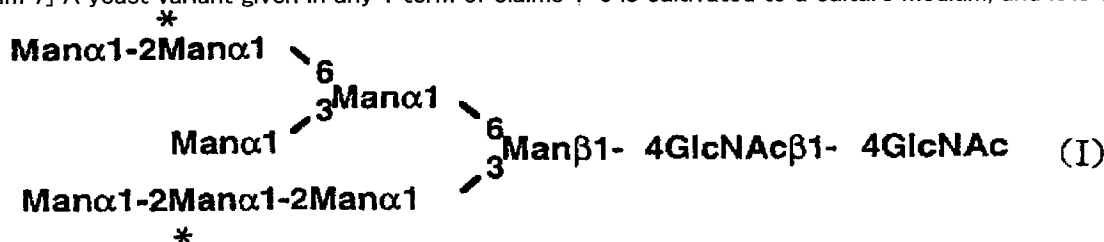
[Claim 3] The yeast variant according to claim 1 or 2 as which the quality of an auxotroph variant is chosen from *ura3* variation, *his3* variation, *leu2* variation, *ade2* variation, *trp1* variation, and *can1* variation.

[Claim 4] The yeast variant according to claim 3 which is the yeast belonging to a *Saccharomyces* (*Saccharomyces*) group.

[Claim 5] The yeast variant according to claim 4 which is the yeast belonging to *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*).

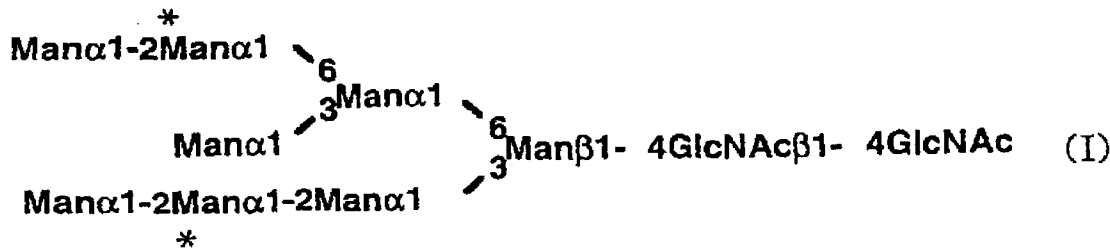
[Claim 6] The yeast variant according to claim 5 which is 19 shares of *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*) TIY.

[Claim 7] A yeast variant given in any 1 term of claims 1-6 is cultivated to a culture medium, and it is the following type in a culture (I) :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.) * A phosphorylation possible part is shown. The manufacturing method of the oligosaccharide chain which is made to carry out generation are recording of the glycoprotein which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.

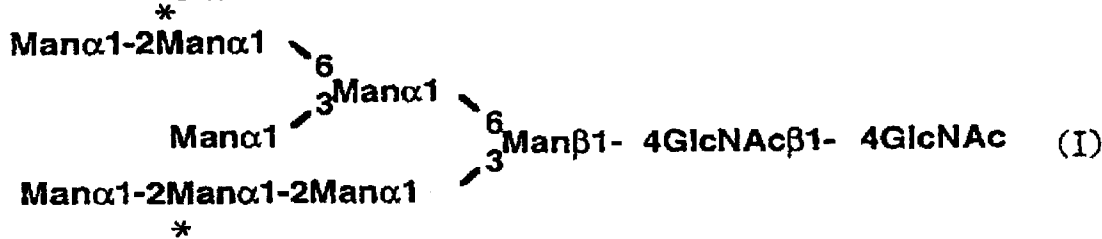
[Claim 8] A yeast variant given in any 1 term of claims 1-6 is cultivated to a culture medium, and it is the following type in a culture (I) :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.) * A phosphorylation possible part is shown.

The manufacturing method of a glycoprotein which is made to carry out generation are recording of the glycoprotein which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein from this culture.

[Claim 9] A yeast variant given in any 1 term of claims 1-6 which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin is cultivated to a culture medium, and it is the following type in a culture (I). :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.) * A phosphorylation possible part is shown.

The manufacturing method of a glycoprotein which is made to carry out generation are recording of the glycoprotein which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein from this culture.

[Claim 10] The yeast variant which introduced at least two or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant with the quality of a variant of och1 variation, mnn1 variation, and mnn4 variation.

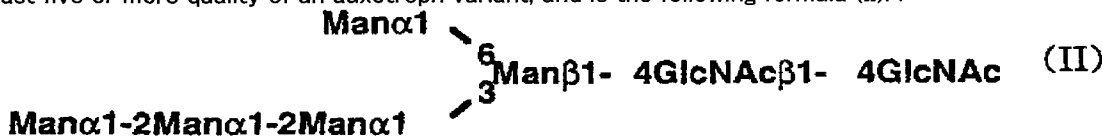
[Claim 11] The yeast variant which introduced at least one or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant given in any 1 term of claims 1-6.

[Claim 12] The manufacturing method of the oligosaccharide chain which cultivates a yeast variant according to claim 10 or 11 to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.

[Claim 13] The manufacturing method of a glycoprotein which cultivates a yeast variant according to claim 10 or 11 to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

[Claim 14] The manufacturing method of a glycoprotein which cultivates the yeast variant according to claim 10 or 11 which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

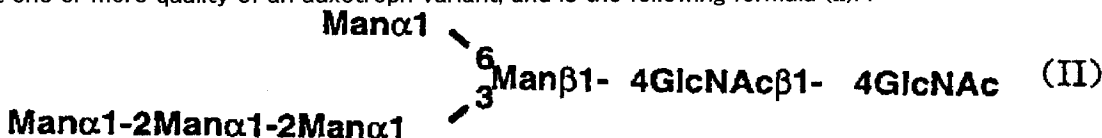
[Claim 15] It is characterized by having the quality of a variant of och1 variation, mnn1 variation, mnn4 variation, and alg3 variation, and at least five or more quality of an auxotroph variant, and is the following formula (II). :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The yeast variant which has the glycoprotein productivity which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain.

[Claim 16] och1 variation which destroyed OCH1 gene, without introducing eventually the gene which carries out the complementation of the auxotroph (deltaoch1), The quality of a variant of mnn1 variation (deltamnn1) which destroyed MNN1 gene, mnn4 variation (deltamnn4) which destroyed MNN4 gene, and alg3 variation (deltaalg3) which destroyed ALG3 gene, It is characterized by having at least one or more quality of an auxotroph variant, and is the following formula (II). :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The yeast variant which has the glycoprotein productivity which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain.

[Claim 17] The yeast variant according to claim 15 or 16 as which the quality of an auxotroph variant is chosen from *ura3* variation, *his3* variation, *leu2* variation, *ade2* variation, *trp1* variation, and *can1* variation.

[Claim 18] The yeast variant according to claim 17 which is the yeast belonging to a *Saccharomyces* (*Saccharomyces*) group.

[Claim 19] The yeast variant according to claim 18 which is the yeast belonging to *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*).

[Claim 20] The yeast variant according to claim 19 which is *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*) YS134-4 A share.

[Claim 21] A yeast variant given in any 1 term of claims 15-20 is cultivated to a culture medium, and it is the following type in a culture



(II). : **Man α 1-2Man α 1-2Man α 1**

(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The manufacturing method of the oligosaccharide chain which is made to carry out generation are recording of the glycoprotein which comes out and contains the ** oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.

[Claim 22] A yeast variant given in any 1 term of claims 15-20 is cultivated to a culture medium, and it is the following type in a culture

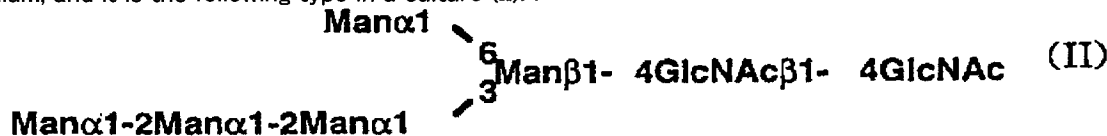


(II). : **Man α 1-2Man α 1-2Man α 1**

(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The manufacturing method of a glycoprotein which is made to carry out generation are recording of the glycoprotein which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein from this culture.

[Claim 23] A yeast variant given in any 1 term of claims 15-20 which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin is cultivated to a culture medium, and it is the following type in a culture (II). :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The manufacturing method of a glycoprotein which is made to carry out generation are recording of the glycoprotein which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein from this culture.

[Claim 24] The yeast variant which introduced at least two or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant with the quality of a variant of *och1* variation, *mnn1* variation, *mnn4* variation, and *alg3* variation.

[Claim 25] The yeast variant which introduced at least one or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant given in any 1 term of claims 15-20.

[Claim 26] The manufacturing method of the oligosaccharide chain which cultivates a yeast variant according to claim 24 or 25 to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.

[Claim 27] The manufacturing method of a glycoprotein which cultivates a yeast variant according to claim 24 or 25 to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

[Claim 28] The manufacturing method of a glycoprotein which cultivates the yeast variant according to claim 24 or 25 which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

[Claim 29] The yeast stock which an alpha-mannosidase II gene is introduced and has alpha-mannosidase II activity.

[Claim 30] The manufacturing method of the alpha-mannosidase II which cultivates a yeast stock according to claim 29 to a culture medium, and is characterized by extracting the alpha-mannosidase II by which generation are recording was carried out into the culture.

[Translation done.]

* NOTICES *

JPO and INPIT are not responsible for any damages caused by the use of this translation.

- 1.This document has been translated by computer. So the translation may not reflect the original precisely.
- 2.**** shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]

Technical field This invention relates to the approach of manufacturing a sugar chain and a glycoprotein by the sugar chain engineering technique using the new yeast variant which has the glycoprotein productivity which added the sugar chain which a mammals cell produces, and the sugar chain which has the same sugar chain structure to proteinic asparagine residue, and this variant.

Background technique There are two kinds of protein which exists in a natural community, the simple protein which consists only of amino acid, and the conjugated protein which the sugar chain, a lipid, a phosphoric acid, etc. combined, and it is known about cytokine that the most is a glycoprotein. Among these, about erythropoietin (EPO) or a tissue plasminogen activator (TPA), if the sugar chain is removed, stopping showing original bioactive will have been clarified (the Kobata positive, a protein nucleic-acid enzyme, 36,775-788 (1991)). Although it is expected that the sugar chain is bearing the role important for the manifestation of bioactive, since correlation with the structure of a sugar chain and bioactive is not necessarily clear, development of the technique which can carry out alteration control of the structures (the class of sugar, a joint location, chain length, etc.) of the sugar chain added to a protein part freely is needed.

It divides roughly into the sugar chain of a glycoprotein, there are an Asn joint mold, a mucin mold, an O-GlcNAc mold, a GPI support mold, a proteoglycan mold, etc. in it (the Takeuchi sincerity, the glycobiology series 5, the Glico technology, the volume Akira Kobata, Hakomori *****, and for Katsutaka Nagai, Kodansha scientific, 191-208 (1994)), it has the biosynthetic path of a proper, respectively, and the physiological function according to individual is borne. Among these, about the biosynthetic path of an Asn joint mold sugar chain, there is many knowledge and it is analyzed in detail.

The precursor which consists of N-acetyl glucosamine, a mannose, and a glucose is compounded on lipid carrier intermediate field, and the biosynthesis of an Asn joint mold sugar chain is first transferred to it by the specific array (Asn-X-Ser or Thr) of a glycoprotein by the endoplasmic reticulum (ER). Next, M8 high mannose mold sugar chain (Man8GlcNAc2) which consists of mannose 8 residue and N-acetyl glucosamine 2 residue is compounded in response to processing (cutting of glucose residue and specific mannose residue).

Although the protein containing this high mannose mold sugar chain is conveyed to a Golgi body and various qualification is received, the qualification with this Golgi body differs greatly by yeast and the mammals (1426 Gemmill, T.R., Trimble, R.B., Biochim.Biophys.Acta., 227 (1999)).

In a mammals cell, in many cases, the alpha-mannosidase I acts on a high mannose mold sugar chain, and the number residue of mannoses is cut. The sugar chain (Man5-8GlcNAc2) generated in this process is a sugar chain called a high mannose mold. N-acetyl GURUKOSAMI nil transferase (GnT) I acts on M5 high mannose mold sugar chain (Man5GlcNAc2) with which 3 residue of mannoses was cut, 1 residue of N-acetyl glucosamines is transferred, and the sugar chain which consists of GlcNAcMan5GlcNAc2 generates. Thus, the made sugar chain is called a hybrid (hybrid) mold. Furthermore, if the alpha-mannosidase II and GnT-II act, it will become the sugar chain structure called a compound (complex) mold called GlcNAc2Man3GlcNAc2, the glycosyltransferase group which also amounts to about ten sorts will act after this, N-acetyl glucosamine, a galactose, a sialic acid, etc. will be added, and various mammals mold sugar chains will be formed (drawing 1). the mammals -- a high mannose mold, a hybrid mold, and a compound die -- although any sugar chain is seen, the sugar chain to combine changes with protein, and the sugar chain with which molds differ also within one protein has joined together. These sugar chains show the function which was [property / the biosynthesis of a glycoprotein, intracellular sorting, antigenic concealment, stability in the living body, / organ targeting] excellent with the class of the mold or united sugar chain (the Endo ****, glycoengineering, industrial Investigation Committees, 64-72 (1992)).

The importance of the sugar chain is pointed out about the erythropoietin used as the first glycoprotein mold drugs in history produced considering the gene recombination body motion object cell as a host. Although the sugar chain of erythropoietin worked in inhibition for association with an acceptor, maintenance of activity structure and an improvement of a moving state in the living body have a decisive contribution, and it was shown that it is indispensable to the manifestation of pharmacological activity as a whole (Takeuchi and Kobata, Glycobiology, 1,337-346 (1991)). Furthermore, functionality strong between the structure of a sugar chain, a class, and a degree (the number of branching formed of GlcNAc combined with Man3GlcNAc2) and the pharmacology effectiveness of erythropoietin was found out (Takeuchi et al., Proc.Natl.Acad.Sci.USA, 86, 7819-7822 (1989)). By the erythropoietin from which branching structure has not developed, the path clearance in a kidney is brought forward and it is reported to be the main causes of this phenomenon that the residence time in the living body becomes short as a result (Misaizu et al., Blood, 86, 4097-4104 (1995)). The example similar to this is seen also by blood serum glycoproteins, such as fetuin, if a galactose is exposed by removing the sialic acid of the end of a sugar chain, it will be recognized by the lectin on the front face of hepatocyte, and disappearing promptly out of blood is found out (Ashwell and Harford, Annu.Rev.Biochem., 51,531-554(1982);Morell et al., J.Biol.Chem., 243,155-159 (1968)).

moreover, if the biosynthesis of many of enzyme groups which carry out localization to Homo sapiens lysosome is carried out and it is conveyed to a Golgi body, a phosphoric-acid radical will add it to the 6th place of the mannose residue of the nonreduction end of the high mannose mold sugar chain -- having -- this -- lysosomal enzyme -- it becomes a specific recognition marker. And after sorting out from other protein, being carried to prelysosome through association with the mannose-6-phosphate acceptor (MPR) which is the high affinity receptor and dissociating from MPR under acid conditions, it is further conveyed to lysosome (von Figura and Hasilik, Annu.Rev.Biochem., 54,167-193 (1984)). this lysosomal enzyme -- when the addition reaction of a specific phosphoric-acid radical is performed by two sorts of enzyme reactions and it has a genetic defect in these genes, abnormalities arising in the targeting device to lysosome, and producing the critical symptoms named a lysosomal disease generically is known (Leroy and DeMars, Science, 157,804-

806 (1967)). Therefore, even if it says to a mammals mold sugar chain and a mouthful, it can be said that the structure is greatly concerned with a function.

On the other hand, in yeast, the mannan mold sugar chain (outside sugar chain) which 100 or more residue of mannoses added to M8 high mannose mold sugar chain from number residue is generated. It is thought that the biosynthesis of the outside sugar chain in *Saccharomyces* group yeast advances in drawing 2 and a path as shown by 3 (Ballou et al., *Proc.Natl.Acad.Sci.USA*, 87, 3368-3372 (1990)). That is, the extended initiation reaction which a mannose adds to M8 high mannose mold sugar chain by α -1 and 6 association first occurs (drawing 2, reactions I and B). It is clear that the enzyme which performs this reaction is protein by which a code is carried out to OCH1 gene (Nakayama et al., *EMBO J.*, 11, 2511-2519 (1992)). Furthermore, when the reaction (drawing 2, II) which carries out stepwise elongation of the mannose by α -1 and 6 association occurs, Pori α -1 and 6 joint mannose association used as the frame of an outside sugar chain are formed (drawing 2, E). Branching of α -1 and the mannose combined two times exists in the mannose of this α -1 and 6 association (drawing 2, 3:C, F, H), and further α -1 and the mannose combined three times may add to the point of the mannose of this α -1 that branched, and 2 association (drawing 2, 3:D, G, H, I). Addition of the mannose of this α -1 and 3 association is based on MNN1 gene product (Nakanishi-Shindo et al., *J.Biol.Chem.*, 268, 26338-26345 (1993)). Moreover, it turns out that the acid sugar chain which the mannose-1-phosphoric acid added to the high mannose mold sugar chain part and the outside sugar chain part is also generated (drawing 2, *; phosphorylation possible part corresponding to * in said formula (I)). It turned out that this reaction is based on the gene in which MNN6 gene carries out a code (Wang et al., *J.Biol.Chem.*, 272, 18117-18124 (1997)), and became clear [the gene (MNN4) which carries out the code of the protein which just controls this rearrangement reaction further] (Odani et al., *Glycobiology*, 6,805-810(1996);Odani et al., *FEBS letters*, 420,186-190 (1997)). In many cases, an outside sugar chain generates a heterogeneous protein product, and purification of protein is made difficult or it reduces specific activity (Bekkers et al., *Biochim.Biophys.Acta*, 1089, 345-351 (1991)). Furthermore, since the structures of a sugar chain differ greatly, the same bioactive as the thing of the mammals origin is not detected, or the glycoprotein produced with yeast has strong immunogenicity to a mammals animal etc. Thus, as a host at the time of making the useful glycoprotein of the mammals origin produce, yeast is made unsuitable. Development of the yeast which can produce a glycoprotein with bioactive equivalent to the thing of the mammals origin, i.e., the glycoprotein containing the sugar chain of a mammals mold, is desired from an institute or the industrial world.

Therefore, it becomes important to isolate the variant which has a sugar chain biosynthesis system which a reaction which adds many mannoses which are qualification of a glycoprotein sugar chain peculiar to the above yeast first does not start in order to produce a mammals mold sugar chain using yeast, but an outside sugar chain stops adding, and sugar chain composition stops with M8 high mannose mold sugar chain. Next, it should be attained by introducing the biosynthesis system gene of a mammals mold sugar chain into M8 high mannose mold sugar chain which is the precursor of this mammals mold sugar chain at the above-mentioned yeast variant. Then, in order to obtain the glycoprotein in which the outside [before] sugar chain carried out deletion, the activity of the deficit stock of the sugar chain product enzyme group outside yeast has been considered. In order to obtain a deficit stock, there are a case where a gene mutation stock is acquired by drugs, UV irradiation, and spontaneous mutation, and the approach of destroying a target gene artificially.

There are reports various until now about the former. For example, mnn2 variant has a deficit in the step of branching which produces α -1 and 2 association from α -1 of an outside sugar chain, and 6 frame, and mnn1 variant has a deficit in the step which generates the mannose of α -1 and 3 association at the head of branching. However, since there is no deficit in α -1 and 6 mannose association which are the frame of an outside sugar chain, these variants all generate chain length's long outside sugar chain. Moreover, although mnn 7, 8, and 9, ten variants, etc. are isolated as a variant in which only 4-15 molecule extent has α -1 and 6 mannose association Also in these variants, an outside sugar chain only becomes short. It is what sugar chain expanding stops with a high mannose mold sugar chain. There is nothing ** (Ballou et al., *J.Biol.Chem.*, 255, 5986-5991(1980);Ballou et al., *J.Biol.Chem.*, 264, 11857-11864 (1989)). As for the addition deficit of an outside sugar chain, secretion variants, such as sec18 from which the protein transport to a Golgi body from an endoplasmic reticulum became temperature sensitivity, are also observed. However, in a sec variant, since the proteinic secretion itself will be checked at an elevated temperature, for the object of the secretory production of a glycoprotein, it is not suitable.

Therefore, it is thought that these variants are unsuitable as host yeast for generating a mammals sugar chain since the high target mannose mold sugar chain cannot be biosynthesized thoroughly.

On the other hand, the sugar chain biosynthetic path in the endoplasmic reticulum (ER) in yeast isolated the variant to which the biosynthesis suffered a loss in various phases, and has been clarified by analyzing this biochemically. Since the alg (asparagine-linked glycosylation) variant became less than the wild type cell in which incorporation of [3H] mannose to a sugar chain has an outer chain, the breakage and extinction by the radiation were isolated by the skillful sorting-out method **** condenses a **** variant. alg3 variation accumulates Dol-pp-GlcNAc2-Man5 (Dol-pp is a dolichol pyrophosphoric acid) under non-permissive temperature in it (906 Tanner, W.et al., *Biochim.Biophys.Acta.*, 8199 (1987)). Moreover, **** and others is analyzing using the deltaoch1mnn1alg3 Mie variant (**** et al., a protein nucleic-acid enzyme, Vol.39, No.4, p.657 (1994)). When the mannan protein sugar chain was analyzed after the fluorescence label by PA (2 amino pilus gene), the principal component showed two peaks which are in agreement with Man8GlcNAc2-PA and Man5GlcNAc2-PA. Among these, it became clear that the former is the same as that of α -1, 2-mannosidase digestion, the result of FAB-MS, etc. to ER core sugar chain. On the other hand, dyad clearance of the Man was carried out by α -1 and 2 mannosidase digestion, the latter produced Man2GlcNAc2-PA, and Man was removed only for one molecule by the processing (partial acetolysis) which cuts specifically α -1 and Man combined six times. From these results, it became clear that the sugar chain of Man5GlcNAc2-PA which this Mie variant produces was the imperfect core mold sugar chain structure shown in said formula (II). in addition, the property of alg3 variation which accumulates Man5GlcNAc2-pp-Dol on a dolichol pyrophosphoric acid as a reason for generating not only Man5GlcNAc2 but Man8GlcNAc2 by this Mie variant — Leakey (leaky) — it is a sake.

On the other hand, about the latter, the deficit stock which destroyed two or more target genes can be built now by development of the gene engineering-technique in recent years.

What has auxotrophic mutation in yeast is known, and there are leu2 variation, trp1 variation, ura3 variation, ade2 variation, his3 variation, etc. as auxotrophic mutation (the work edited by Taiji Oshima, the biochemistry laboratory procedure 39, a yeast molecular genetics laboratory procedure, 119-144 (1996)). If the gene of the origin which does not have variation is introduced, these auxotroph can be canceled, and growth will become possible even if it does not add a need component into a culture medium. Gene disruption of

yeast can be performed based on this principle (drawing 4). By this approach, the structure which make carry out fragmentation or partial deletion of the target gene DNA on a plasmid, and inserts the selective marker gene DNA suitable there by actuation within a test tube first and by which the selective marker was sandwiched between the upper section of a target gene and a downstream is produced. next, a line with this structure — it permutes by introducing DNA into a yeast cell by the DNA construct which put the lifting and the selective marker for two recombination between the analogous segments of the ends of an introductory fragment, and the target gene on a chromosome (Rothstein, Methods Enzymol., 101,202–211 (1983)). By this approach, one selective marker is needed for destroying one gene.

The molecular breeding of the yeast stock which suffered a loss in the outside sugar chain is already indicated by JP,6–277086,A and JP,9–266792,A. However, it turned out that the acid sugar chain which has phosphoric-acid residue in the glycoprotein sugar chain produced by the duplex variant (deltaoch1deltamnn1) indicated by JP,6–277086,A is contained. Homo sapiens etc. is the structure which does not exist in the sugar chain of the mammals origin, and this acid sugar chain is recognized to be a foreign matter in a mammalian body, and is considered that antigenic is shown (Ballou, Methods Enzymol., 185,440–470 (1990)). Then, the 4-fold variant (it indicates to JP,9–266792,A) which destroyed the function of a mannose transferring enzyme gene (KRE2) to perform the lenthening reaction of the gene (MNN4) which just controls mannose–1–phosphate transfer further, and an O–joint mold sugar chain was built. The sugar chain of the glycoprotein which the yeast stock indicated by this produces became clear [having the target M8 high mannose mold sugar chain]. Furthermore, it is clear the stock's which introduced alpha–1 of the Aspergillus saitoi origin and a 2–mannosidase gene into this destructive yeast to have the high mannose mold sugar chain (Man5–8GlcNAc2) with which 1– number residue cutting of the mannose was carried out (Chiba et al., J.Biol.Chem., 273, 26298–26304 (1998)).

moreover Niima and others further alg3 variation The 4-fold variant of the introduced another kind it is creating (Shimma Y.et al., Mol.Gen.Genet., 256,469–480(1997);Wang et al., J.Biol.Chem.272, 18117–18124(1997); Yoichi Niima, and **** Yoshifumi —) The collection of the 32nd yeast genetics forum summaries, p. 64(1999);Shimma Y.et al., Abstracts ofXIX International Conference On Yeast Genetics and Molecular Biology, p.443 (1999).

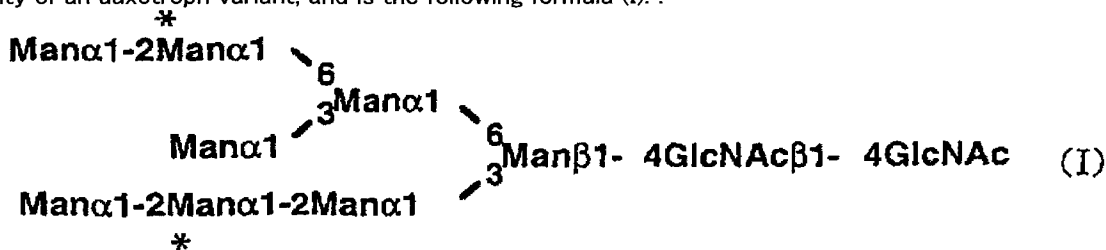
However, it is expected by the sugar chain of the glycoprotein which these yeast stocks produce that the glycoprotein containing a high mannose mold sugar chain does not show activity equivalent to the glycoprotein produced from the mammals cell from the sugar chain structure so that clearly also from the example of erythropoietin, although it exists also in the mammals and does not have antigenic. Moreover, the activity of this 4-fold variant after four selective markers (leu2, ura3, lys2, trp1) of the host who is a recessive gene variation becomes impossible by destruction of a target gene. Furthermore, about one of the remaining auxotroph markers, not mutation but since it is destroyed artificially, inclusion of a up to [the yeast chromosome using homology with these marker loci on a chromosome] cannot be performed. Therefore, it is difficult to introduce two or more genes belonging to a sugar chain hydrolase gene cluster required to produce the sugar chain of a mammals mold, a glycosyltransferase gene cluster, and a sugar nucleotide transporter gene cluster, and genes for performing production of a useful glycoprotein to these yeast stocks. As mentioned above, it is known that there are about ten glycosyltransferase groups required to produce the sugar chain of a mammals mold, and it is thought that it is unsuitable to make this yeast cell into a host in order to carry out alteration control of the sugar chain structure satisfactorily.

The technical problem of this invention conquers the above–mentioned problem in production with the yeast of an Asn–joint mold glycoprotein, and is to offer the approach of manufacturing the glycoprotein containing a sugar chain with the same sugar chain structure as the high mannose mold which Homo sapiens and other mammals cells produce, a hybrid mold, and a compound die, and this sugar chain using yeast.

Disclosure of invention this invention persons making the auxotrophic mutation which is a selective marker hold, as a result of repeating research wholeheartedly that the above–mentioned technical problem should be solved Namely, the inside of the gene which biosynthesizes a specific outside sugar chain in yeast, without introducing eventually the gene which carries out the complementation of the auxotroph, alpha–1 which performs the extended addition reaction of initiation, the gene which carries out the code of the 6 mannosyltransferase (OCH1), alpha–1 which adds a mannose to the nonreduction end of a sugar chain, the gene which carries out the code of the 3 mannosyltransferase (MNN1), And according to the new yeast variant (auxotroph Mie variant) which destroyed the gene (MNN4) which controls addition of a mannose–1–phosphoric acid It found out that various mammals mold sugar chains were producible by that a sugar chain with the same sugar chain structure as a mammals mold sugar chain is producible, and introducing the biosynthesis system gene of a mammals mold sugar chain into this variant.

That is, this invention relates to the following (1) – (14).

(1) It is characterized by having the quality of a variant of och1 variation, mnn1 variation, and mnn4 variation, and at least four or more quality of an auxotroph variant, and is the following formula (I). :



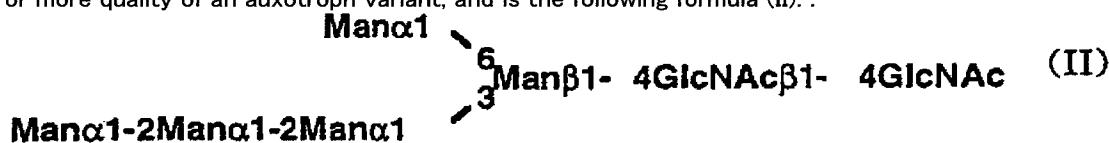
(Man shows a mannose among a formula and GlcNAc shows N–acetyl glucosamine.) * A phosphorylation possible part is shown.

The yeast variant which has the glycoprotein productivity which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain.

(2) och1 variation which destroyed OCH1 gene, without introducing eventually the gene which carries out the complementation of the auxotroph (deltaoch1), The quality of a variant of mnn1 variation (deltamnn1) which destroyed MNN1 gene, and mnn4 variation (deltamnn4) which destroyed MNN4 gene, The yeast variant which has the glycoprotein productivity which contains the oligosaccharide chain which is characterized by having at least one or more quality of an auxotroph variant, and is expressed with the above–mentioned formula (I) as an asparagine joint mold sugar chain.

(3) The above (1) as which the quality of an auxotroph variant is chosen from ura3 variation, his3 variation, leu2 variation, ade2 variation, trp1 variation, and can1 variation, or a yeast variant given in (2).

- (4) A yeast variant given in the above (3) which is the yeast belonging to a *Saccharomyces* (*Saccharomyces*) group.
- (5) A yeast variant given in the above (4) which is the yeast belonging to *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*).
- (6) A yeast variant given in the above (5) which is 19 shares of *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*) TIY.
- (7) The above (1) Manufacturing method of the oligosaccharide chain which cultivates a yeast variant given in either of - (6) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains the oligosaccharide chain expressed with the above-mentioned formula (1) in a culture as an asparagine joint mold sugar chain, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.
- (8) The above (1) Manufacturing method of a glycoprotein which cultivates a yeast variant given in either of - (6) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains the oligosaccharide chain expressed with the above-mentioned formula (I) in a culture as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein from this culture.
- (9) the mammals -- the origin -- an asparagine -- association -- a mold -- a glycoprotein -- a code -- carrying out -- a gene -- containing -- recombination -- a plasmid -- a transformation -- carrying out -- having made -- the above -- (-- one --) -- (-- six --) -- either -- a publication -- yeast -- a variant -- a culture medium -- cultivating -- a culture -- inside -- the above -- a formula -- (-- I --) -- expressing -- having -- an oligosaccharide -- a chain -- an asparagine -- association -- a mold -- a sugar chain -- ***** -- containing -- a glycoprotein -- generation -- are recording -- carrying out -- making -- this -- a culture -- from -- this -- a glycoprotein -- extracting -- things -- the description -- ** -- carrying out -- a glycoprotein -- a manufacturing method .
- (10) The yeast variant which introduced at least two or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant with the quality of a variant of och1 variation, mnn1 variation, and mnn4 variation.
- (11) The above (1) Yeast variant which introduced at least one or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant given in either of - (6).
- (12) The manufacturing method of the oligosaccharide chain which cultivates a yeast variant the above (10) or given in (11) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.
- (13) The manufacturing method of a glycoprotein which cultivates a yeast variant the above (10) or given in (11) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.
- (14) The manufacturing method of a glycoprotein which cultivates a yeast variant the above (10) which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin, or given in (11) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.
- The gene to which this invention persons do the code of the above-mentioned yeast alpha-1 and the 6 mannosyotransferase again (OCH1), alpha-1 which adds a mannose to the nonreduction end of a sugar chain, the gene which carries out the code of the 3 mannosyotransferase (MNN1), To and the above-mentioned yeast variant (auxotroph Mie variant) which destroyed the gene (MNN4) which controls addition of a mannose-1-phosphoric acid Furthermore, according to the new yeast variant (four-fold auxotroph variant) which destroyed the gene (ALG3) which participates in the sugar chain biosynthesis in ER Alpha-mannosidase which is one of the biosynthesis system genes of a mammals mold sugar chain It found out that various mammals mold sugar chains were producible by introducing the biosynthesis system gene of other mammals mold sugar chains, without introducing II gene.
- That is, this invention relates to the following (15) - (30) further.
- (15) It is characterized by having the quality of a variant of och1 variation, mnn1 variation, mnn4 variation, and alg3 variation, and at least five or more quality of an auxotroph variant, and is the following formula (II) . :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

The yeast variant which has the glycoprotein productivity which comes out and contains the oligosaccharide chain with which it is expressed as an asparagine joint mold sugar chain.

- (16), without introducing eventually the gene which carries out the complementation of the auxotroph och1 variation (deltaoch1) which destroyed OCH1 gene, mnn1 variation which destroyed MNN1 gene (deltamnn1), The quality of a variant of mnn4 variation (deltamnn4) which destroyed MNN4 gene, and alg3 variation (deltaalg3) which destroyed ALG3 gene, The yeast variant which has the glycoprotein productivity which contains the oligosaccharide chain which is characterized by having at least one or more quality of an auxotroph variant, and is expressed with the above-mentioned formula (II) as an asparagine joint mold sugar chain.
- (17) The above (15) characterized by choosing the quality of an auxotroph variant from ura3 variation, his3 variation, leu2 variation, ade2 variation, trp1 variation, and can1 variation, or a yeast variant given in (16).
- (18) A yeast variant given in the above (17) which is the yeast belonging to a *Saccharomyces* (*Saccharomyces*) group.
- (19) A yeast variant given in the above (18) which is the yeast belonging to *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*).
- (20) A yeast variant given in the above (19) which is *Saccharomyces cerevisiae* (*Saccharomyces cerevisiae*) YS134-4 A share. (21) The above (15) Manufacturing method of the oligosaccharide chain which cultivates a yeast variant given in either of - (20) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains the oligosaccharide chain expressed with the above-mentioned formula (II) in a culture as an asparagine joint mold sugar chain, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.
- (22) The above (15) Manufacturing method of a glycoprotein which cultivates a yeast variant given in either of - (20) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains the oligosaccharide chain expressed with the above-mentioned formula (II) in a culture as an asparagine joint mold sugar chain, and is characterized by extracting this glycoprotein

from this culture.

(23) the mammals -- the origin -- an asparagine -- association -- a mold -- a glycoprotein -- a code -- carrying out -- a gene -- containing -- recombination -- a plasmid -- a transformation -- carrying out -- having made -- the above -- (-- 15 --) -- (-- 20 --) -- either -- a publication -- yeast -- a variant -- a culture medium -- cultivating -- a culture -- inside -- the above -- a formula -- (-- II --) -- expressing -- having -- an oligosaccharide -- a chain -- an asparagine -- association -- a mold -- a sugar chain -- ***** -- containing -- a glycoprotein -- generation -- are recording -- carrying out -- making -- this -- a culture -- from -- this -- a glycoprotein -- extracting -- things -- the description -- ** -- carrying out -- a glycoprotein -- a manufacturing method . (24) The yeast variant which introduced at least two or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant with the quality of a variant of och1 variation, mnn1 variation, mnn4 variation, and alg3 variation.

(25) The above (15) Yeast variant which introduced at least one or more biosynthesis system genes of a mammals mold sugar chain into the yeast variant given in either of -- (20).

(26) The manufacturing method of the oligosaccharide chain which cultivates a yeast variant the above (24) or given in (25) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by collecting these oligosaccharide chains from this culture from the glycoprotein which extracted and extracted this glycoprotein.

(27) The manufacturing method of a glycoprotein which cultivates a yeast variant the above (24) or given in (25) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

(28) The manufacturing method of a glycoprotein which cultivates a yeast variant the above (24) which carried out the transformation in the recombination plasmid containing the gene which carries out the code of the asparagine joint mold glycoprotein of the mammals origin, or given in (25) to a culture medium, is made to carry out generation are recording of the glycoprotein which contains an oligosaccharide chain as an asparagine joint mold sugar chain in a culture, and is characterized by extracting this glycoprotein from this culture.

(29) The yeast stock which an alpha-mannosidase II gene is introduced and has alpha-mannosidase II activity.

(30) The manufacturing method of the alpha-mannosidase II which cultivates a yeast stock given in the above (29) to a culture medium, and is characterized by extracting the alpha-mannosidase II by which generation are recording was carried out into the culture.

This description includes the content indicated by the description of No. 233215, and/or the drawing the Japan Heisei 11 patent application which is the foundation of the priority of this application.

The easy explanation array number 1 of an array table shows the primer A for amplifying 5' field of MNN1 gene.

The array number 2 shows the primer B for amplifying 5' field of MNN1 gene.

The array number 3 shows the primer C for amplifying 3' field of MNN1 gene.

The array number 4 shows the primer D for amplifying 3' field of MNN1 gene.

The array number 5 shows the primer E for amplifying 3' field of MNN4 gene.

The array number 6 shows the primer F for amplifying 3' field of MNN4 gene.

The array number 7 shows the primer G for amplifying 5' field of MNN4 gene.

The array number 8 shows the primer H for amplifying 5' field of MNN4 gene.

The array number 9 shows the primer I for amplifying 5' field of ALG3 gene.

The array number 10 shows the primer J for amplifying 5' field of ALG3 gene.

The array number 11 shows the primer K for amplifying 3' field of ALG3 gene.

The array number 12 shows the primer L for amplifying 3' field of ALG3 gene.

The array number 13 is alpha-mannosidase. The primer M for amplifying the amino terminal side field of II gene is shown.

The array number 14 is alpha-mannosidase. The primer N for amplifying the amino terminal side field of II gene is shown.

The array number 15 is alpha-mannosidase. The primer O for amplifying the center-section field of II gene is shown.

The array number 16 is alpha-mannosidase. The primer P for amplifying the center-section field of II gene is shown.

The array number 17 is alpha-mannosidase. The primer Q for amplifying the C terminal side field of II gene is shown.

The array number 18 is alpha-mannosidase. The primer R for amplifying the C terminal side field of II gene is shown.

The array number 19 shows the array S of the double stranded DNA which carries out the code of the gene combined so that HA-tag may be made to repeat 3 times.

The array number 20 shows the array T of the double stranded DNA which carries out the code of the transmembrane domain of OCH1 gene.

The array number 21 is alpha-mannosidase. The primer U for amplifying a part of catalyst field of II gene is shown.

The array number 22 is alpha-mannosidase. The primer V for amplifying a part of catalyst field of II gene is shown.

The array number 23 is Homo sapiens UDP-GlcNAc. The primer W for amplifying a Transporter gene is shown.

The array number 24 is Homo sapiens UDP-GlcNAc. The primer X for amplifying a Transporter gene is shown.

The array number 25 is Homo sapiens prepro. The primer Y for amplifying alpha-factor and a FGF gene is shown.

The array number 26 is Homo sapiens prepro. The primer Z for amplifying alpha-factor and a FGF gene is shown.

Gestalt for inventing This invention is hereafter explained to a detail.

The quality of a variant required for the yeast variant of this invention is the variation of an outside sugar chain biosynthesis system gene peculiar to yeast, and is specifically och1 variation, mnn1 variation, mnn4 variation or och1 variation, mnn1 variation, mnn4 variation, and alg3 variation.

That is, as long as it has the above-mentioned variation, it may be a spontaneous mutation stock or you may be an artificial mutation stock.

Moreover, the quality of an auxotroph variant for introducing the foreign gene in the yeast variant of this invention is specified in the yeast stock to be used, and is specifically chosen from ura3 variation, his3 variation, leu2 variation, ade2 variation, trp1 variation, and can1 variation. Although the number of the quality of an auxotroph variant is based on the number of the genes to introduce, generally one quality of an auxotroph variant is required to introduce one gene. Since the gene fragment to introduce is long, and introductory effectiveness falls, as a result manifestation effectiveness also falls when introducing two or more genes, much quality of an auxotroph variant is needed, so that there are many introductory genes.

In this invention, "the gene which carries out the complementation of the auxotroph" is a gene of the synthetic system of biogenic substances, such as amino acid and a nucleic acid. Since close requires variation on which, as for the quality of a variant, these genes do not function, the gene which carries out the complementation is the gene itself on which origin functions. Therefore, the gene of the original yeast stock origin is desirable.

Moreover, although used for that (the quality of a variant is introduced) which destroys one or the gene beyond it with "without it introduces eventually the gene which carries out the complementation of the auxotroph", the selective marker of an auxotroph variant, i.e., the quality, beyond one piece or it, in case the characteristic concerned of the number of destructive genes and the same number remains and after destruction is gene disruption for the second time, it says that the same characteristic concerned can be used repeatedly (refer to drawing 5).

The yeast variant (the following, auxotrophic mutant) which holds the quality of an auxotroph variant for introducing a foreign gene in this invention and by which the outside sugar chain biosynthesis system gene peculiar to yeast was destroyed is producible as follows. First, isolation of a DNA gene fragment required for destruction of a target gene By the genome project of *Saccharomyces cerevisiae* the conformation of all on the chromosome is known (Goffeau et al., Nature, and 387 (suppl.) — 1-105 (1997) — from things U.S. ATCC () [American Type] Culture It is possible to receive distribution of the gene fragment which contains near the target gene from public engines, such as Collection, (ATCC Recombinant DNA materials, 3rd edition, 1993). Moreover, it is possible by extracting genomic DNA from *S.cerevisiae* according to the general technique, and sorting out the object gene. In the above, the extract of genomic DNA can be performed according to Cryer's and others approach (Methods in Cell Biology, 12, 39-44 (1975)), and P.Philippsen's and others approach (Methods Enzymol., 194,169-182 (1991)) from *S.cerevisiae*.

After making a target gene amplify by the PCR method, it performs gene disruption. Although the PCR method is the technique which can amplify the specific fragment of DNA to hundreds of thousands or more times in about 2 - 3 hours using the combination of the sense antisense primer of the ends of the field, heat-resistant DNA polymerase, a DNA magnification system, etc. by in vitro (inch vitro) one, the synthetic single stranded DNA of 25-30mer is used as a primer, and it uses genomic DNA for magnification of a target gene as mold.

Setting to this invention, destruction of a target gene is Rothstein and Methods. It can carry out by following to Enzymol. and the approach indicated by 101,202-211 (1983) fundamentally. This approach produces the structure which make carry out fragmentation or partial deletion of the target gene DNA on a plasmid, and inserts the selective marker gene DNA suitable there first and by which the selective marker was sandwiched between the upper section of a target gene, and a downstream, next introduces this structure into a yeast cell. The target gene on a lifting and a chromosome is permuted in an introductory fragment in two recombination by the above actuation between the analogous segments of the ends of an introductory fragment (DNA structure which put the selective marker), and the target gene on a chromosome.

Concretely, it explains taking the case of production of a MNN1 gene-disruption stock. The cassette of hisG-URA3-hisG is cut down with a restriction enzyme from the plasmid (Alani et al., Genetics, 116,541-545 (1987)) by which the hisG gene DNA fragment of a salmonella built by Alani and others is combined with the ends of URA3 gene, it inserts in the target gene on a plasmid, and the destroyed allele is built. It permutes by the target gene of a chromosome using this plasmid, and a gene disruption stock is obtained. URA3 gene inserted in the chromosome is pinched by hisG, and falls out from a chromosome with hisG of 1 copy by homonous recombination during a hisG array. Although the hisG fragment of 1 copy remains and is destroyed still more by the target gene on a chromosome, a host cell serves as a Ura phenotype (drawing 5). 5-fluoro orotic acid (5-FOA) can perform homonous recombination between this hisG. It becomes impossible to grow the cell strain to which ura3 variant is resistance (Boeke et al., Mol.Gen.Genet., 197,345-346(1984);Boeke et al., Methods Enzymol., 154,165-174 (1987)), and has a Ura3+ phenotype in 5-FOA to a 5-FOA culture medium. therefore, the culture medium which added 5-FOA — resistance — if a stock with a characteristic is separated, the actuation using URA3 is possible again.

Hereafter, the auxotroph Mie variant (deltaoch1deltamnn1deltamnn4) made into the object of this invention can be obtained by performing MNN4 gene disruption and OCH1 gene disruption in this MNN1 gene-disruption stock by the same technique. Furthermore, by performing ALG3 gene disruption by the same technique, the four-fold auxotroph variant (deltaoch1deltamnn1deltamnn4deltaalg3) made into the object of this invention can be obtained.

Therefore, on "the artificial destructive stock" which performed gene disruption artificially by the above-mentioned technique, the quality of an auxotroph variant which has the original yeast stock is not spoiled by gene disruption actuation. Therefore, even if the number of the quality of an auxotroph variant which this artificial mutation stock has is the Mie variant and it is 4-fold variant, it is equal to the number of the quality of an auxotroph variant which the original yeast stock has, and it will have it at least one or more pieces. Since the above-mentioned technique is not used on the "spontaneous mutation stock" to which it was not based on the above-mentioned technique, but gene disruption has happened automatically on the other hand, it is unrelated to the change in the number of the quality of an auxotroph variant.

If it creates using the yeast stock which holds six quality of an auxotroph variant although OCH1, MNN1, and MNN4 gene are destroyed based on a conventional method when creating the yeast variant which produces M8 high mannose sugar chain in this invention, the number of the quality of an auxotroph variant which this variant has since only three quality of an auxotroph variant does not remain will become at least four or more pieces.

Moreover, although the spontaneous mutation stock of mnn1 variation and alg3 variation can be used in creation by the conventional method when creating further the yeast variant which produces M8 high mannose sugar chain which variation produced at ALG3 gene to OCH1, MNN1, and MNN4 gene variation, since it is necessary to destroy OCH1 and MNN4 gene further, two quality of an auxotroph variant will be used. Therefore, if the yeast stock which holds the six above-mentioned quality of an auxotroph variant is used, since, as for the quality of an auxotroph variant, only four pieces will remain, the number of the qualities of an auxotroph variant of this variant becomes at least five or more pieces.

In addition, as a selective marker, not only auxotroph markers, such as URA3, but the marker which gives resistance to drugs, such as G418, cerulenin, OLE OBASHIJIN, ZEOSHIN, canavanine, a cycloheximide, and a tetracycline, can be used by the above-mentioned actuation. Moreover, it is also possible to perform installation and destruction of a gene by using as a marker the gene which gives metal ion resistance over solvent resistance, glycerol, a salt, etc. to a methanol, ethanol, etc., such as osmotic-pressure resistance and copper, etc.

When using phage as a general approach, for example, a vector, as the approach of the transformation by the installation and this to the

cell of DNA in the above-mentioned actuation, a host can be made to incorporate DNA efficiently by the approach of infecting this with an *Escherichia coli* host etc. moreover, the method of changing into the condition of processing by lithium salt and being easy to incorporate DNA as an approach of carrying out the transformation of the yeast using a plasmid automatically, and making a plasmid incorporate — or the approach of introducing DNA into intracellular electrically is employable (Becker and Guarente, *Methods Enzymol.*, 194,182-187 (1991)).

Moreover, each of isolation, purification, etc. of DNA in the above-mentioned actuation can refine DNA the case of a conventional method, for example, *Escherichia coli*, with the DNA extract by alkali / the SDS method, and ethanol precipitate and also RNase processing, PEG settling, etc. Moreover, the decision of the DNA array of a gene etc. can be made by the usual approach (Sanger et al., *Proc.Natl.Acad.Sci.*, USA, 74, 5463-5467 (1977)), for example, a dideoxy chain termination method etc. Furthermore, the decision of the above-mentioned DNA sequence can be easily made also by using a commercial sequence kit etc.

Although the auxotrophic mutant produced as mentioned above can produce the mammals mold sugar chain of a high mannose mold, in order to make the sugar chain mammals mold sugar chain of a hybrid mold and a compound die produce further, it introduces a sugar chain hydrolase gene cluster peculiar to yeast, and a glycosyltransferase gene cluster into this variant. Moreover, only a minute amount exists in the organ to which the biosynthesis of the sugar chain is actually carried out even if there is no sugar nucleotide transporter of these within yeast although it is required for the sugar nucleotide which serves as a raw material of a sugar chain since the biosynthesis of a sugar chain is originally performed within ER and a Golgi body as mentioned above to exist in these organs, or it is. Therefore, the sugar nucleotide transporter gene cluster which moves from cytoplasm the sugar nucleotide by which the biosynthesis was carried out into ER and a Golgi body is still more nearly required of intracytoplasmic.

Therefore, in this invention, the gene belonging to the above-mentioned sugar chain hydrolase gene cluster, a glycosyltransferase gene cluster, and a sugar nucleotide transporter gene cluster is called "biosynthesis system gene of a mammals mold sugar chain."

As a sugar chain hydrolase gene cluster, genes, such as alpha-mannosidase (alpha-mannosidase I, alpha-mannosidase II), As a glycosyltransferase gene cluster, N-acetyl GURUKOSAMI nil transferase (GnT-I, GnT-II, GnT-III, GnT-IV, GnT-V), As genes, such as galactosyltransferase (GalT) and fucosyltransferase (FucT), and a sugar nucleotide transporter gene cluster UDP-GlcNAc Transporter, UDP-Gal Genes, such as Transporter, are mentioned. These genes are possible also by also being able to isolate and use the gene of the mammals origin and compounding a gene.

The above-mentioned "biosynthesis system gene of a mammals mold sugar chain" introduces only a number required to produce the target sugar chain for the gene belonging to one sort or two sorts or more of above-mentioned gene clusters. When the gene to introduce is plurality, even if it belongs to the gene cluster with those genes of the same kind, you may belong to the gene cluster of a different kind mutually.

If the variant which introduced the above-mentioned foreign gene group into an above-mentioned auxotrophic mutant or this above-mentioned auxotrophic mutant is cultivated to a culture medium The high mannose mold sugar chain which the content of an outside sugar chain peculiar to yeast falls, and a mammals cell produces (Man5-8GlcNAc2), The glycoprotein containing the same Asn joint mold sugar chain as a hybrid mold sugar chain (GlcNAcMan5GlcNAc2) and a compound-die sugar chain (Gal2GlcNAc2Man3GlcNAc2 grade) can be made to produce out of yeast intracellular or a cell.

When using the Mie variant (deltaoch1deltamnn1deltamnn4) as an auxotrophic mutant, specifically It is alpha-mannosidase to this variant. By introducing I gene and a GnT-I gene A hybrid mold sugar chain can be made to produce, and it is the biosynthesis system gene (alpha-mannosidase it II(s)) of a mammals mold sugar chain. GnT-II, GalT, UDP-GlcNAc Transporter, UDP-Gal A double strand compound-die sugar chain (Gal2GlcNAc2Man2GlcNAc2) can be made to produce by introducing a Transporter gene.

Furthermore, a 3 chain compound-die sugar chain and a 4 chain compound-die sugar chain can also be made to produce by introducing GnT-IV and a GnT-V gene.

Moreover, it is alpha-mannosidase when using 4-fold variant (deltaoch1deltamnn1deltamnn4deltaalg3) as an auxotrophic mutant. A double strand compound-die sugar chain (Gal2GlcNAc2Man2GlcNAc2) can be made to produce by introducing the biosynthesis system gene (alpha-mannosidase I, GnT-I, GnT-II, Gal-T, UDP-GlcNAc Transporter, UDP-Gal Transporter gene) of a mammals mold sugar chain, without introducing II gene.

In order to obtain the sugar chain to generate and a glycoprotein by high yield, it is desirable to carry out a high manifestation for the above-mentioned enzyme in a suitable organ (for example, Golgi body). Therefore, it is effective to use the gene doubled with the codon usage of yeast. Moreover, in order to carry out localization of the enzyme to a suitable organ, it is also effective to add the signal sequence of yeast etc. Although how to use vectors, such as 2-micrometer plasmid type (YE_p type) and a chromosome inclusion mold type (YI_p type), about installation of a gene can be considered, according to the object, it can use properly. Since a YE_p type vector can introduce a gene by many copies, a large quantity can be made to discover a gene. On the other hand, since a YI_p type vector can make a gene exist in a chromosome, it can hold the gene stably. In order to make a gene discover, especially a need promotor has a desirable configuration-manifestation promotor to production of a sugar chain, although the induction manifestation promotor of configuration-manifestation promotors, such as GAPDH and PGK, GAL1, and CUP1 grade etc. is not limited. However, since growth of yeast may be affected for sugar chain hydrolase, a glycosyltransferase, and a sugar nucleotide transporter gene one sort or when two or more shots are made to express, it is necessary to take into consideration an induction promotor's activity, and the sequence which introduces a gene in that case.

Moreover, the variant obtained by drugs, UV irradiation, and spontaneous mutation in addition to the variant obtained by the artificial gene destructive procedure is also contained in the auxotrophic mutant in this invention like the above. This spontaneous mutation stock can produce similarly the glycoprotein which has a mammals mold sugar chain and a mammals mold sugar chain by introducing the biosynthesis system gene (gene belonging to a sugar chain hydrolase gene cluster, a glycosyltransferase gene cluster, and a sugar nucleotide transporter group) of the above-mentioned mammals mold sugar chain.

furthermore, in order to make the glycoprotein with the above-mentioned sugar chain of the different-species living thing origin produce The gene which connected the genes (cDNA etc.) which carry out the code of the target glycoprotein by making the above-mentioned yeast variant into a host to a promotor's lower stream of a river which can be discovered with yeast is produced. or [including in the above-mentioned yeast host by homologous recombination] — or By inserting in a plasmid and carrying out the transformation of the above-mentioned host, a glycoprotein to have been produced out of yeast intracellular or a cell is recoverable by producing the above-mentioned host's transformant and cultivating this by the well-known approach.

Culture of the above-mentioned yeast variant can be performed according to the conventional method commonly used by culture of

yeast. For example, the synthetic medium (a carbon source, a nitrogen source, mineral, amino acid, a vitamin, etc. are included) except the amino acid whose supply adds various kinds of culture-medium components supplied from Difco, and is attained with a marker required for the duplicate and maintenance of a plasmid etc. can be used (Sherman, *Methods Enzymol.*, 194, 3-57 (1991)).

What is necessary is just to use isolation of usual protein and a purification method, in order to carry out isolation purification of the glycoprotein from the above-mentioned culture (culture medium, culture biomass).

For example, after culture termination, centrifugal separation recovers a cell, a cell is crushed by the ultrasonic crusher, the French press, the MANTONGAURIN homogenizer, dynamill, etc. after suspending in the drainage system buffer solution, and a cell-free extract is obtained. The isolation purification method of protein usual from the supernatant liquid obtained by carrying out centrifugal separation of this cell-free extract, namely, the salting-out method by the solvent extraction method, an ammonium sulfate, etc., the desalting method, settling by the organic solvent, The anion-exchange chromatography method using resin, such as diethylaminoethyl (DEAE)-sepharose, S-Sepharose The cation-exchange chromatography method using resin, such as FF (Pharmacia manufacture), The hydrophobic chromatography method using resin, such as butyl sepharose and phenyl sepharose, Gel filtration, His using molecular sieving The affinity chromatography method using Bind resin (product made from Novagen) etc., independent in technique, such as electrophoresis methods, such as the chromatofocusing method and isoelectric focusing, — or it can combine and use and a purification preparation can be obtained.

Example An example explains this invention concretely hereafter. However, these examples do not limit the technical range of this invention at all.

[Example 1] Mammals mold sugar chain productivity The yeast variant which it has Production and the property of breeding (1) *deltamnn1* auxotrophic mutant of a (*deltamnn1deltamnn4deltaoch1* auxotroph Mie variant) From pNK51 (Alani et al., *Genetics*, 116,541-545 (1987)) which already has a report The cassette (HUH) which the salmonella *hisG* gene has combined with the ends of URA3 gene by the direct repeat was cut by BglII and BamHI, and was inserted in the BamHI part of the *Escherichia coli* plasmid pSP73. This plasmid was named pSP73-HUH.

MNN1 gene is located near the yeast chromosome kinetochore of No. 5, and the DNA sequence of MNN1 gene is registered into the GenBank database by L23753 (Yip et al., *Proc.Natl.Acad.Sci.USA*, 9, 2723-2727 (1994)). 3' field was amplified for 5' field of MNN1 gene by PCR using Primer C (GCATGCTACATAACTCCAATCAGCAGCAATATGTC: array number 3) and Primer D (GCGGCCGCGTGTCTCTGTTCCGGTAACGTTTAAACCAAT: array number 4), respectively, using Primer A (GGATCCGAAGAAAACCTAATACATTGAAGT: array number 1) and Primer B (GCATGCCCTTTGGTTTAATAATCTCCGGAGTGC: array number 2). A nest and *PHYHdeltamnn1* were produced to the SphI part of Plasmid *PHYH* which has HIS3 marker for these DNA fragments. In order to destroy MNN1 gene using a HUH cassette, the SphI fragment of 1.8Kb was acquired from *PHYHdeltamnn1*, and pSP73-*deltamnn1::HUH* inserted in the SphI part of pSP73-HUH was built. By cutting this plasmid by the NotI part, linearization was carried out and the transformation of the wild strain W303-1A (MATa *leu2-3,112his 3-11*, 15 *ade2-1 ura3-1 trp1-1 can 1-100*) was carried out using the acetic-acid lithium method (Ito et al., *J.Bacteriol.*, 153,163-168 (1983)). It wound around the plate of a SD-Ura (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and uracil 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained.

From the transformant, genomic DNA was prepared, and it checked that the uracil marker was built into the chromosome of MNN1 field by the PCR method, and was referred to as one share of TIY.

This strain was selected in the YSD culture medium (1% yeast extract, 2% glucose, an adenine (40 mg/L), uracil (20 mg/L)) containing 5-FOA, and the URA3 gene omission stock was obtained. The *mnn1* destructive stock which omitted URA3 gene using the PCR method like the above-mentioned approach was checked. *deltamnn1*: This strain containing :*hisG* was set to three shares of TIY(s).

Since the MNN1 destructive stock is missing in alpha-1 of a nonreduction end, and the mannose of three association, it is known that the mobility of the invertase which receives N-joint mold qualification will become earlier than a wild strain. The wild strain cultivated in the YPAD culture medium and three shares of TIY(s) were re-suspended in the nutrition culture medium (1% yeast extract, 2%Bacto peptone, adenine (40 mg/L)) which contains sucrose 0.2%, respectively, and were cultivated for 3 hours. After it suspended in the SDS sample buffer after collecting biomasses, and the glass bead ground, SDS polyacrylamide electrophoresis was performed 6% using supernatant liquid. Detection of an invertase performed activity staining after induction using the triphenyl tetrazolium by sucrose (Ballou, *Methods Enzymol.*, 185,440-470 (1990)). Consequently, the invertase of three shares of TIY(s) to produce checked that mobility was earlier than the thing of a wild strain.

(2) Production and the property of a *deltamnn1deltamnn4* auxotroph duplex variant MNN4 gene is located in yeast the chromosome of No. 11, and the DNA sequence of MNN4 gene is registered into the GenBank database by D83006 (Odani et al., *Glycobiology*, 6,805-810 (1996)). 5' field was amplified for 3' field of MNN4 gene by PCR using Primer G (CCCCCGAATTCAAGTCGGAGAACCTGACTG: array number 7) and Primer H (ATGGGCCCCACTAGTATGCATCTCGCGTGGCATGG: array number 8), respectively, using Primer E (AGATGCATACTAGTGGGCCATTGTGATTGGAAT: array number 5) and Primer F (CCCCCGAATTCGTGTGAAGGAATAGTGACG: array number 6). A nest and pSP73-*deltamnn4::HUH* were produced to said EcoRI part of pSP73-HUH which has a HUH cassette for these DNA fragments. By cutting this plasmid by the SpeI part, linearization was carried out and the transformation of three shares of TIY(s) was carried out using the acetic-acid lithium method. It wound around the plate of a SD-Ura culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained.

From the transformant, genomic DNA was prepared, and it checked that the uracil marker was built into the chromosome of MNN4 field by the PCR method, and was referred to as nine shares of TIY(s).

This strain was selected in the YSD culture medium containing 5-FOA, and the URA3 gene omission stock was obtained. The *mnn4* destructive stock which omitted URA3 gene using the PCR method like the above-mentioned approach was checked. *deltamnn1*: This strain containing :*hisG* *deltamnn4::hisG* was set to 11 shares of TIY(s).

The existence of the phosphoric-acid radical in a sugar chain can be dyed in various colors by Alcian Blue (alcian blue). Electrification of Alcian Blue is just carried out, and it is coloring matter combined with negative electrification. Then, if a yeast cell is suspended in the buffer solution of pH3 and 0.1% of Alcian Blue 8GX (the product made from Sigma, code No.A3157) is added, in a sugar chain, only a thing with a phosphoric-acid radical dyes blue, and does not have a phosphoric-acid radical white. Since the sugar chain of a cell cortex has almost no phosphoric-acid radical, a *deltamnn4* destructive stock is not dyed Alcian Blue. As a result of cultivating a wild strain, and TIY3 and TIY11 in a nutrition culture medium and arsine blue's dyeing each cell, it checked that only TIY11 did not dye blue.

(3) Production and the property of a *deltamnn1deltamnn4deltaoch1* auxotroph Mie variant OCH1 gene is located in yeast the chromosome of No. 7, and the DNA sequence of OCH1 gene is registered into the GenBank database by D11095 (Nakayama et al., EMBO J., 11, 2511-2519 (1992)). The interior AatII-HpaI part of OCH1 gene of pBL-OCH1 (Nakayama et al., EMBO J., 11, 2511-2519 (1992)) containing the overall length of OCH1 gene already built was cut, and pBL-delta och1::HUH which inserted the HUH cassette obtained from flush-end-ized pNKY51 was produced. By cutting this plasmid by SalI and BamHI, the field containing deltaoch1::HUH was started and the transformation of TIY11 was carried out by the acetic-acid lithium method. A stock including deltaoch1 destruction is after a transformation and 0.3M, in order to show hyposmosis susceptibility. It wound around the plate of the SD-Ura culture medium containing KCl, and cultivated for two days at 30 degrees C, and the transformant was obtained.

From the transformant, genomic DNA was prepared, and it checked that the uracil marker was built into the chromosome of deltaOCH1 field by the PCR method, and was referred to as 17 shares of TIY(s).

About this strain, it is 5-FOA and 0.3M. It selected in the YSD culture medium containing KCl, and the URA3 gene omission stock was obtained. The och1 destructive stock which omitted URA3 gene using the PCR method like the above-mentioned approach was checked. *deltamnn1*: This strain containing :hisG *deltamnn4*::hisG *deltaoch1*::hisG was set to 19 shares of TIY(s).

19 shares of this auxotroph Mie variant TIY is the trust number FERM on July 27, Heisei 11 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken). International deposition is carried out as BP-6802.

In order that 19 shares of books TIY including och1 destruction may form a high mannose mold sugar chain, it is known that the mobility of an invertase will become early compared with a wild strain, three shares of TIY(s), and 11 shares of TIY(s). Then, in order to confirm the effectiveness in the sugar chain length of an och1 destructive stock, as a result of detecting an invertase by the wild strain and the approach shown above from TIY3, TIY11, and 19 shares of TIY(s), respectively, it checked that mobility was a wild strain and the order of TIY3, TIY11, and TIY19 early.

[Example 2] Production and the property of the yeast variant (delta four-fold *mnn1deltamnn4deltaoch1deltaalg3* auxotroph variant) which has mammals mold sugar chain productivity ALG3 gene is located in yeast the chromosome of No. 2, and the DNA sequence of ALG3 gene is registered into the GenBank database by Z35844 (Feldmann et al., EMBO J., 13, 5795-5809 (1994)). 3' field was amplified for 5' field of ALG3 gene by PCR using Primers K (GAATTCCTATCCACCAAACCTCAAGCAAGCA: array number 11) and L (GCGGCCGCCGAGAGGGTGAACGGTGCAACTCAGGATT: array number 12), respectively, using Primers I (GCGGCCGCCGAGACCTGAATCTTCGACACGCAAGAAAAA: array number 9) and J (GAATTCGCTTTTGAACAAAATCAAAAGGGGCATAAC: array number 10). A nest and pSP73-*alg3*::HUH were produced to the EcoRI part of pSP73-HUH which has a HUH cassette for these DNA fragments. By cutting this plasmid by the NotI part, linearization was carried out and the transformation of 19 shares of TIY(s) was carried out using the acetic-acid lithium method. It wound around the plate of a SD-Ura culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained.

From the transformant, genomic DNA was prepared, and it checked that the uracil marker was built into the chromosome of ALG3 field by the PCR method, and was referred to as 134 shares of YS(s).

This strain was selected in SD culture medium containing 5-FOA, and the URA3 gene omission stock was obtained. The *alg3* destructive stock which omitted URA3 gene using the PCR method like the above-mentioned approach was checked. *deltamnn1*: This strain containing :hisG *deltamnn4*::hisG *deltaoch1*::hisG *deltaalg3*::hisG was used as the YS134-4 A share.

This four-fold auxotroph variant YS134-4 A share is the trust number FERM on July 27, Heisei 11 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken). International deposition is carried out as BP-6801.

Since this YS134-4 A share including *alg3* destruction has short sugar chain length, it is known that the mobility of an invertase will become early compared with a wild strain, three shares of TIY(s), 11 shares of TIY(s), and 19 shares of TIY(s). Then, in order to confirm the effectiveness in the sugar chain length of an *alg3* destructive stock, as a result of detecting an invertase by the approach shown in the example 1 (1) from a wild strain, three shares of TIY(s), 11 shares of TIY(s), 19 shares of TIY(s), and YS134-4 A share, respectively, it checked that mobility was the order of a wild strain, three shares of TIY(s), 11 shares of TIY(s), 19 shares of TIY(s), and YS134-4 A share early.

[Example 3] Separation and structural analysis of a content sugar chain of the cell-cortex mannan protein from a *deltamnn1deltamnn4deltaoch1* auxotroph Mie variant Concanavalin A is lectin C-3, C-4, and the hydroxyl group of the C-6th place indicate compatibility to be to the sugar chain containing 2 or more residue of non-permuted alpha-D-Man, and glucan, a chitin, etc. which are yeast cell wall carbohydrates, and mannan protein can be separated by fixing this to a column. First, the mannan protein of a cell cortex was separated from the biomass of 19 shares of TIY(s) (Peat et al., J.Chem.Soc., 29 (1961)).

0.3M 50ml of YPAD culture media containing KCl was put into the 500-ml Sakaguchi flask, it cultivated at 30 degrees C for 24 hours, biomasses were collected according to centrifugal separation, it suspended in the 10ml 100mM sodium-citrate buffer solution (pH7.0), and 121 degrees C was heated in the autoclave for 1 hour. After cooling, centrifugal separation was carried out, supernatant liquid was taken, similarly, the solid heated, and 10ml water is added once again and it collected [centrifugal separation of it was carried out and] supernatant liquid. All extracts were doubled and it poured into the ethanol of the amount of 3 times. The produced white precipitate was dried. This was dissolved in the buffer solution for concanavalin A (ConA) columns (0.1M sodium phosphate buffer solution containing 0.15M sodium chloride and a 0.5mM calcium chloride (pH7.2)), the ConA-agarose column (0.6 x 2cm, HONEN Corporation make) was presented, and it was eluted with the buffer solution for ConA columns which contains alpha-methyl mannoside of 0.2M after washing with the buffer solution for ConA columns. It freeze-dried by having dialyzed the obtained fraction, and mannan protein was obtained.

Next, to the obtained mannan protein, enzyme processing was performed and the Asn joint mold sugar chain was cut down. That is, the freeze-drying preparation was dissolved in the buffer solution for N-glycosidase F of 100microl (0.5% SDS, 0.1M Tris-HCl buffer solution containing 0.35% 2-mercaptoethanol (pH8.0)), and boiling processing was carried out for 5 minutes. 7.5% after returning to a room temperature of 50microl Nonidet H2O of P-40,138microl, N-glycosidase of 12microl F (product made from BERINGA) was added and 37 degrees C was processed for 16 hours. BioRad In AG501-X8 column, equivalent phenol:chloroform (1:1) was added after demineralization, it shook violently, a surfactant and protein were removed, and it considered as the sugar chain preparation. The following actuation was performed in order to carry out fluorescent labeling (it is called pyridylamino-izing and PA-ization) of the

obtained sugar chain. After concentration hardening by drying, the coupling reagent (552mg 2-aminopyridine) was dissolved in the acetic acid of 200microl of 40microl was added and sealed, and 90 degrees C of sugar chain preparations were processed for 60 minutes. After returning to a room temperature, the reduction reagent (200mg borane dimethylamine complex) was dissolved in the acetic acid of H₂O of 50microl and 80microl of 140microl was added and sealed, and was processed 80 degrees C for 80 minutes. After the reaction, after adding 200ml of aqueous ammonia, phenol:chloroform (1:1) was added so that it might become ana further, and the water layers which shake violently and contain PA-ized oligosaccharide were collected. This was repeated 7 times and unreacted 2-aminopyridine was removed. It filtered with the 0.22-micrometer filter about supernatant liquid, and considered as PA-ized oligosaccharide preparation. In HPLC using an amide column, it is possible to separate PA-ized oligosaccharide by the chain length. A column is TSKGel. Using Amide-80 (4.6 x 250mm, TOSOH make), the solvent prepared the mixed liquor (A liquid) of 35:65 of the 200mM acetic-acid-triethylamine buffer solution (pH7.0) and an acetonitrile, and the mixed liquor (B liquid) of 50:50 of the 200mM acetic-acid-triethylamine buffer solution (pH7.0) and an acetonitrile.

By pouring Solvent A by rate-of-flow 1.0 ml/min beforehand, the column was equilibrated, the rate of Solvent B was linearly raised from immediately after sample impregnation to 50% over 25 minutes, and the sink and PA-ized oligosaccharide were eluted for 5 minutes in Solvent A and Solvent B after that with 50:50. The result is shown in drawing 6. The sugar chain of the mannan protein of 19 shares of TIY(s) which are a deltaoch1deltamnn1deltamnn4 auxotroph Mie variant to produce was mainly one peak in the amide column. This peak was in agreement with the elution location of a Man8GlcNAc2-PA preparation (TAKARA SHUZO make). Therefore, it became clear that the high mannose mold sugar chain of Man8GlcNAc2 has added to the mannan protein of 19 shares of TIY(s) to produce.

[Example 4] Alpha-mannosidase to a deltaoch1deltamnn1deltamnn4 auxotroph Mie variant Installation of I gene Alpha-mannosidase which manages the initiation reaction in order to biosynthesize the sugar chain near a mammals mold with yeast What is necessary is to introduce the gene of I (alpha-1, 2-mannosidase) into an auxotroph Mie variant, and just to make it discover. Thereby, a high mannose mold sugar chain can biosynthesize Man5GlcNAc2 sugar chain which is the precursor of the mammals hybrid mold which became still shorter, or a mammals compound die.

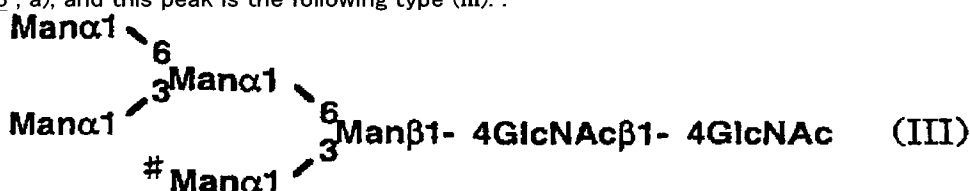
The endoplasmic reticulum mold manifestation plasmid pGAMH1 (Chiba et al., J.Biol.Chem., 273, 26298-26304 (1998)) of alpha-1 of the Aspergillus saitoi origin which already has a manifestation track record, and 2-mannosidase was used, and the transformation of 19 shares of TIY(s) was carried out by the acetic-acid lithium method. What carried out the transformation as control only by the vector pG3 which does not contain alpha-1 and a 2-mannosidase gene was used. It wound around the plate of a SD-Trp (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and tryptophan 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained. The obtained transformant was set to TIY19pGAMH1.

Preparation and analysis by HPLC were performed for the sugar chain like the example 3 from the obtained transformant.

The analysis result by the amide column was shown in drawing 7. In the thing of only the vector of control, like the result of an example 3, it is mainly one peak (drawing 7, a), and was in agreement with the elution location of a Man8GlcNAc2-PA preparation (TAKARA SHUZO make). On the other hand, in TIY19pGAMH1 containing alpha-1 and a 2-mannosidase gene, four peaks were mainly seen (drawing 7, b). Man5GlcNAc2-PA, Man6GlcNAc2-PA, Man7GlcNAc2-PA, the Man8GlcNAc2-PA preparation, and the elution location of these peaks corresponded with the early order of elution. These are called the high mannose mold sugar chain of a Homo sapiens mold. Next, elution isolated the earliest Man5GlcNAc2-PA fraction preparatively, and presented the opposite phase column.

In HPLC using an opposite phase column, it is possible to separate PA-ized oligosaccharide according to the structure. A column is TSKGel. Using ODS-80TM (4.6 x 150mm, TOSOH make), a solvent is the 100mM ammonium acetate buffer solution (pH4.0) (A liquid) and 0.5%. The 100mM ammonium acetate buffer solution (pH4.0) (B liquid) containing 1-butanol was prepared.

By passing what mixed Solvent A and Solvent B by 95:5 beforehand by rate-of-flow 1.2 ml/min, the column was equilibrated, the rate of Solvent B was linearly raised from immediately after sample impregnation to 50% over 20 minutes, and PA-ized oligosaccharide was eluted. The result is shown in drawing 8. The sugar chain fraction isolated preparatively is mainly one peak in an opposite phase column (drawing 8, a), and this peak is the following type (III). :



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.) # A GnT-I site of action is shown.

It was in agreement with the elution location of the Man5GlcNAc2-PA preparation (TAKARA SHUZO make) which comes out and has the structure expressed (drawing 8, b). Therefore, it became clear that the sugar chain of the Man5GlcNAc2 mold which is the precursor of a hybrid mold and a compound die is contained in the mannan protein of one share of TIY19pGAMH to produce.

[Example 5] Composition of a hybrid mold sugar chain (GlcNAcMan5GlcNAc2) preparation The object sugar chain was first compounded using the enzyme reaction of GnT-I out of the cell for check examination of the biosynthesis of a hybrid mold sugar chain (GlcNAcMan5GlcNAc2). The substrate specificity of GnT-I is dramatically strict, and transferring GlcNAc only to the mannose residue of the location of # by beta-1 and 2 association to the sugar chain structure shown by said formula (III) is known.

The manifestation with the yeast of a rat GnT-I gene was successful with Yoshida and others (Yoshida et al., Glycobiology, 9, 53-58 (1999)). After connecting this gene to the lower stream of a river of the GAP-DH promotor of pG3 which is a multicopy plasmid, it cut by SmaI-NaeI and the field containing a promotor, and ORF of GnT-I following it and a terminator was started. Next, this fragment was introduced into the SmaI part of pYO354 which is a multicopy plasmid. This plasmid was named pYOG4. The transformation of 500 shares of wild type yeast YPH was carried out by the acetic-acid lithium method using this plasmid. It wound around the plate of a SD-Trp (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and tryptophan 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained. The obtained transformant was set to YPH500/pYOG4.

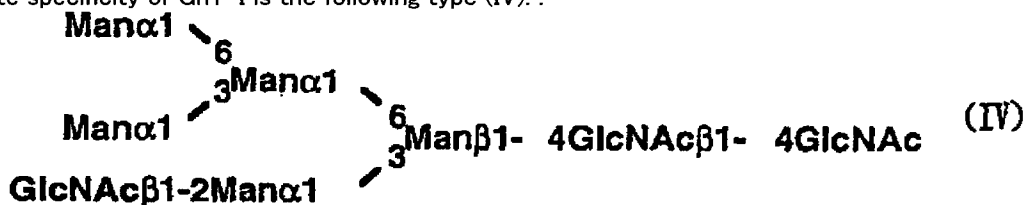
With the 500ml SD-Trp (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and tryptophan 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) solution, liquid culture of this was carried out and it carried out the harvest. It

suspends after washing with chilled water in 5.7ml (50mM(s) potassium phosphate containing 1M sorbitol (pH7.5)) of spheroplast culture media, and they are 2-mercaptoethanol 9microl and 12mg Zymolyase. 100T were dissolved in the spheroplast culture medium of 300microl, and, in addition, 30 degrees C kept it warm for 45 minutes. 1M sorbitol 15ml was added, after centrifugal, again, by 1M sorbitol 15ml, it washed and the harvest of the precipitate was carried out. It is lysis to this precipitate. 4ml (a 250mM sorbitol, 2microg [/ml] antipain, 2microg [/ml] chymostatin, 3microg [/ml] leupeptin, 3microg [/ml] pepstatin, 1mM bends amidine, 1mM EDTA, 1mM EGTA, 10mM(s) triethanolamine (pH7.2) solution containing 1mM PMSF) of buffer(s) was added, the cell was destroyed with the homogenizer, centrifugal was carried out by 220xg, and supernatant liquid was collected. Centrifugal [of this supernatant liquid] is carried out to a pan by 100,000xg(s), and it is lysis about that precipitate fraction. buffer It suspended in 150microl and considered as the enzyme solution of GnT-I. In addition, other GnT activity was not detected by this preparation.

Next, the object sugar chain was compounded. Man5GlcNAc2 sugar chain (it purchases from TAKARA SHUZO) by which the label was carried out by PA was made into the acceptor substrate, and this was poured distributively in the tube by 200pmol(s). GnT-I enzyme solution 8.2microl prepared above in this tube after evaporation to dryness, 0.2M MnCl2 2microl and GnT-I reaction buffer (0.17M MES (pH6.0), 1.7% Triton X-100, 0.34% Bovine Serum Albumin, 8.47mM AMP, 1.69mM UDP-GlcNAc, 169mM GlcNAc) 9.8microl were added, and 37 degrees C was made to react for 3 hours. It boiled for 5 minutes, and filtered with the 0.22-micrometer filter after the reaction halt, and HPLC was presented.

A column is TSKGel. Using ODS-80TM (4.6 x 250mm, TOSOH make), a solvent is 0.15%. The 100mM ammonium acetate buffer solution (pH6.0) containing 1-butanol was used. By pouring a solvent by rate-of-flow 1.2 ml/min beforehand, the column was equilibrated, the sample was poured in and PA-ized oligosaccharide was eluted. The result is shown in drawing 9. Reactants are mainly two peaks in an opposite phase column, and the peak eluted early was in agreement with the elution location of a Man5GlcNAc2-PA preparation (structure is shown in the TAKARA SHUZO make and said formula (III)). Therefore, it was thought that this was an unreacted acceptor substrate.

About the peak eluted late on the other hand, this was isolated preparatively and mass analysis by TOF-MS was performed after purification. LASERMAT2000 made from ThermoQuest is used and it is 2.5% of 2 and 5-dihydroxybenzoic as a matrix. acid, 40% It analyzed using 0.01% phosphoric-acid disodium containing an acetonitrile. Consequently, the mass of the above-mentioned peak fraction is considerable the bottom to the molecular mass ($m/z=1521(H^+)$; $m/z=1541(Na^+)$) expected. The sugar chain obtained from the strict substrate specificity of GnT-I is the following type (IV).



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

It was thought that it was hybrid mold sugar chain GlcNAcMan5GlcNAc2 for it to be alike and have the shown structure.

[Example 6] Alpha-mannosidase to a deltaoch1deltamnn1deltamnn4 auxotroph Mie variant Installation of I gene and a GnT-I gene What is necessary is to introduce a GnT-I gene into the yeast stock produced in the example 4 further, and just to make it discovered, in order to biosynthesize a hybrid mold sugar chain with yeast. Thereby, mammals hybrid mold GlcNAcMan5GlcNAc2 sugar chain can be biosynthesized.

From the endoplasmic reticulum mold manifestation plasmid pGAMH1 (Chiba et al., J.Biol.Chem., 273, 26298-26304 (1998)) of alpha-1 of the Aspergillus saitoi origin used in the example 4, and 2-mannosidase, it cut by SmaI-NaeI and the field containing a promotor, and ORF of alpha-1 following it and 2-mannosidase and a terminator was started. Next, this fragment was introduced into the SmaI part of pYOG4. This plasmid was named pYOMG4. The transformation of 19 shares of TIY(s) was carried out by the acetic-acid lithium method using this plasmid. As control, what carried out the transformation only by pYO354 was used. It wound around the plate of a SD-Trp (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and tryptophan 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained. The obtained transformant was set to TIY19pYOMG4.

This alpha-mannosidase Four shares of auxotroph Mie variant TIY19pYOMG(s) which I gene and a GnT-I gene are introduced and produce a hybrid mold sugar chain are the trust numbers FERM on July 2, Heisei 11 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken). International deposition is carried out as BP-6775.

Preparation and analysis by HPLC were performed for the sugar chain like the example 3 from the obtained transformant.

The analysis result by the amide column was shown in drawing 10. In the thing of only the vector of control, like the result of an example 3, it is mainly one peak and was in agreement with the elution location of a Man8GlcNAc2-PA preparation (TAKARA SHUZO make) (drawing 10, A). In TIY19pYOMG4 containing alpha-1, a 2-mannosidase gene, and a GnT-I gene, five peaks were mainly seen (drawing 10, B). Man5GlcNAc2-PA, Man6GlcNAc2-PA, Man7GlcNAc2-PA, the Man8GlcNAc2-PA preparation, and the elution location of four of these peaks (drawing 10, B; peak a, c, d, e) corresponded. These are called the high mannose mold sugar chain of a Homo sapiens mold. When only further alpha-1 and a 2-mannosidase gene were introduced, the new peak (drawing 10, B; peak b) which was not seen appeared. The elution location of this peak was in agreement with the elution location of the hybrid mold GlcNAcMan5GlcNAc2 sugar-chain reference standard produced in the example 5. Furthermore this peak was isolated preparatively and the opposite phase column was presented like the example 3. A column, a solvent, and conditions were performed by the approach shown in the example 5. The sugar chain fraction isolated preparatively is mainly one peak in an opposite phase column (drawing 11), and this peak was in agreement with the elution location of a GlcNAcMan5GlcNAc2-PA preparation again. Therefore, it became clear that the sugar chain of the GlcNAcMan5GlcNAc2 mold which is a hybrid mold is contained in the mannan protein of four shares of TIY19pYOMG(s) to produce.

[Example 7] Homo sapiens liver alpha-mannosidase Manifestation with the yeast of II Alpha-mannosidase II is an enzyme which changes a hybrid mold sugar chain into a single strand compound-die sugar chain within a Golgi body.

Homo sapiens liver alpha-mannosidase II gene sequence is registered into the GenBank database by U31520 (Misago et al.,

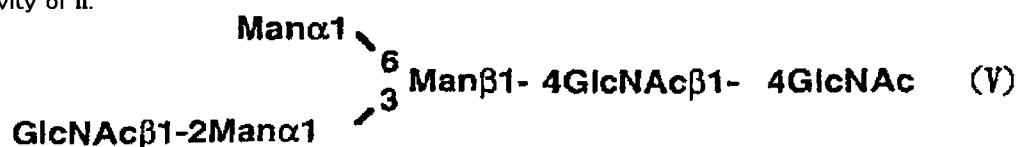
Proc.Natl.Acad.Sci., 92, 11766-11770 (1995)). Human of Clontech Liver Marathon-Ready cDNA is made into a template. Alpha-mannosidase Primers M (CGCCGCCGAGCTCTAAAAAATGAAGTTAAGCCGCC: array number 13) and N (ATCCCACCACTTTGAAAGGT: array number 14) are used for the part which carries out the code of the amino terminal side field of II. Primers O (GAAGACTCACGGAGGAAGTT: array number 15) and P (ATGGCGGTATATGTGCTCGA: array number 16) are used for the part which carries out the code of the center. The part which carries out the code of the C terminal side field was amplified by PCR using Primer Q (CGCAGTTTGGGATACAGCAA: array number 17) and Primer R (ATTATTATTAGCGGCCGCCCTCAACTGGATTG: array number 18), respectively. The obtained DNA fragment is introduced into the SrfI part of pCRScript, and it was made to become the right array which switches by the BglII part and carries out the code of all the fields after checking an array. This plasmid was named pCRMAN2.

It is alpha-mannosidase because of the manifestation check of target protein. The gene combined so that HAtag which consists of 30bp (s) which carry out the code of the Muggle thynnine epitope to the three-dash terminal of II gene to an influenza virus may be repeated 3 times was produced, and the vector was built. That is, chemosynthesis of the double stranded DNA which consists of an array S (array number 19) was carried out, and it inserted between BamHI of pYEX-BX of the plasmid for a manifestation, and an EcoRI part. This plasmid was named pYEX-BX-3HA. Next, it is alpha-mannosidase at pCRMAN2 to BamHI, and EcoRI. The part which carries out the code of the II was started, and it inserted between SacI of pYEX-BX-3HA, and a NotI part. This plasmid was named pYEMAN2-HA. Next, it is alpha-mannosidase in order to raise the amount of manifestations in yeast. The part which carries out the code of the transmembrane domain of II was permuted by the transmembrane domain part of the gene (OCH1) which carries out the code of alpha-1 of yeast, and the 6-mannosyltransferase. That is, chemosynthesis of the double stranded DNA which consists of an array T (array number 20) was carried out, and it inserted between SacI of pBluescript, and an EcoRI part. This plasmid was named pBOCH1. On the other hand, it is alpha-mannosidase, using pYMAN2-HA as a template. A part of part which carries out the code of the catalyst field of II was amplified using Primer U (TTAGACTACCCATGGAACCCGCGCCGCGAGGGCTCCTTC: array number 21) and Primer V (CAGGAGAACTTTGGTTCGAAAAAGCTTTGACTTCTT: array number 22). It cut by NcoI and HindIII after checking this array, and inserted between NcoI of pBOCH1, and a HindIII part. Next, the fragment was cut down by SacI and PstI from this plasmid, and it permuted between ScaI of pYEMAN2-HA, and PstI. This plasmid was named pYEOM2-HA.

The transformation was performed using the acetic-acid lithium method, using the *S.cerevisiae* wild type yeast YPH500 as a host. pYEX-BX-3HA was used as control. It wound around the plate of a SD-Ura (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and uracil 0.67% nucleobase, and amino acid mixture (20-400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained.

To OD 660= 0.8, after culture, the yeast by which the transformation was carried out carried out suitable amount addition, and cultivated the copper sulfate by the SD-Ura culture medium at 30 degrees C for further 2 hours. The cell was crushed with the glass bead in the SDS sample buffer after the harvest, and western blot analysis was performed using the cell extract. Western blot analysis uses rat anti-HA antibody as a primary antibody, and uses anti-rat IgG antibody peroxidase complex as a second antibody, and detection is Super. Signal It carried out by exposing to an X-ray film by making Ultra into a substrate. Consequently, by the cell extract which carried out the transformation by pYEOM2-HA, the signal was checked to a signal not being seen at all in control in the molecular weight about 140000 location (drawing 12).

Next, the hybrid mold sugar chain (structure is shown in a formula (IV)) produced in the example 5 is made into a substrate, and it is alpha-mannosidase. The enzyme activity of II was measured. It is 0.2M to the said tube after drying hybrid mold sugar chain (structure is shown in formula (IV)) 100pmol within a sample tube. MnCl₂ 2 and 1M After 8microl Adding every [2micro / l] and H₂O for GlcNAc and 1M sodium acetate buffer solution (pH5.6), the cell extract was 8microl Added and the enzyme reaction was started. After having boiled after incubation at 37 degrees C overnight, suspending the reaction and removing an insoluble fraction by centrifugal, it analyzed in HPLC. In addition, the conditions of HPLC analysis followed the example 5. Consequently, alpha-mannosidase When the cell extract of yeast which made II discover was made into the source of an enzyme, as compared with control, the peak for 40 minutes was increasing clearly (drawing 13 , B). The peak for these 40 minutes is alpha-mannosidase since it was in agreement with the elution location of the single strand compound-die sugar chain (Oguri et al., J.Biol.Chem., 272, 22721-22727 (1997)) expressed with the following type (V) obtained from the enzyme digest of PA-sugar chain reference standard (TAKARA SHUZO PA-Sugar Chain 022). It checked that it was the activity of II.



(Man shows a mannose among a formula and GlcNAc shows N-acetyl glucosamine.)

[Example 8] Production of the auxotroph *Mie* variant which introduced the gene required to produce a double strand compound-die sugar chain The manifestation with the yeast of *Homo sapiens* GnT-II is reported by Yoshida and others (Yoshida S.et al., Abstracts of the meeting - Yeast Cell Biology, p.279, Cold Spring Harbor Laboratory (1997)). The GnT-II gene field which contains a promotor from this vector pSY114-GnT-II for a manifestation is started by XbaI, and it is pBluescript. It inserted in the XbaI part of SK. This plasmid was named pBlueGT2. Next, from the plasmid pYOG4 shown in the example 6, the GnT-I gene field including a promotor was started by BamHI and XbaI, and it inserted in BamHI of pBlueGT2, and a XbaI part. DNA after cutting down the object fragment by BssHII from this plasmid T four The fragment was inserted in the SmaI part of pASZ10 plasmid (Stotz& Linder, Gene, 95, 91-98 (1990)) which has ADE2 as a marker after graduating an end by polymerase. This plasmid was named pASZGN12. The transformation of 19 shares of auxotroph *Mie* variants TIY which made pASZGN12 the shape of a straight chain by HpaI, and were produced in the example 1 was performed by the acetic-acid lithium method. SD-Ade which contains 0.3M KCl after a transformation (it wound around the plate of a glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and an adenine, and an amino acid mixture (20-400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that GnT-I and a GnT-II gene were included in the chromosome of ADE2 field by the PCR method, and was referred to as 22 shares of YCY(s). Each enzyme activity was measured using the cell extract of 22 shares of YCY(s), and the manifestation of GnT-I and GnT-II was checked.

On the other hand, the manifestation with the yeast of *Homo sapiens* beta-1 and 4-GalT is reported by above-mentioned Yoshida and

others (.). (Yoshida S.etal., Abstracts of the meeting – Yeast Cell Biology, p.279, Cold Spring Harbor Laboratory (1997)) From this vector pGalT13C for a manifestation, beta-1 and a 4-GalT gene field including a promotor were started by Sall and XhoI, and it inserted in Sall of pRS403, and a XhoI part. This plasmid was named pRSGAL1. Moreover, Homo sapiens UDP-Gal The manifestation with the yeast of Transporter (Ugt2p) is reported by Kainuma and others (Kainuma et al., Glycobiology, 9,133-141 (1999)). From plasmid YEp352-GAP-UGT2 for a manifestation of this gene (UGT2), the gene field including a promotor was started by BamHI, and it inserted in the BamHI part of pRSGAL1. This plasmid was named pRSGATP1. pRSGATP1 was made into the shape of a straight chain by NdeI, and the transformation of 22 shares of YCY(s) was performed by the acetic-acid lithium method. SD-His which contains 0.3M KCl after a transformation (it wound around the plate of a glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and a histidine, and an amino acid mixture (20-400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that beta-1, 4-GalT, and UGT2 gene was included in the chromosome of HIS3 field by the PCR method, and was referred to as 42 shares of YCY(s). Each enzyme activity was measured using the cell extract of 42 shares of YCY(s), and the manifestation of beta-1, 4-GalT, and Ugt2p was checked.

Next, Homo sapiens liver alpha-mannosidase From vector pYEOM2-HA for a manifestation of II, the gene fragment which contains HA-tag by SacI and SphI is cut down, and it is DNA. T four The end was graduated by polymerase. This fragment was inserted in the SmaI part of pAUR123. Alpha-mannosidase which contains a promotor after checking being tied to the promotor in the direction of the right II gene field was started by BamHI and it inserted in the BamHI part of pRS406. This plasmid was made into the shape of a straight chain by NdeI, and said transformation of 42 shares of YCY(s) was performed by the acetic-acid lithium method. SD-Ura which contains 0.3M KCl after a transformation (it wound around the plate of a glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and a uracil, and an amino acid mixture (20-400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that the gene was included in the chromosome of URA3 field by the PCR method, and was referred to as 52 shares of YCY(s). Enzyme activity is measured using the cell extract of 52 shares of YCY(s), and it is alpha-mannosidase. The manifestation of II was checked.

alpha-1 shown in the example 4, alpha-1 which includes promoterregion by NaeI and SmaI from the vector pGAMH1 for a manifestation of 2-mannosidase, and a 2-mannosidase gene fragment were cut down, and it inserted in the SmaI part of pYO325 vector. This plasmid was named pYOM5. Furthermore, UDP-GlcNAc required to supply a substrate to a Golgi body The Transporter gene was introduced. Homo sapiens UDP-GlcNAc The manifestation with the yeast of a Transporter gene is reported by Ishida and others (Ishida et al., J.Biochem., 1261, 68-77 (1999)). This vector for a manifestation is made into a template, and it is UDP-GlcNAc by the PCR method at Primer W (AGAGCGGCCGCAAAATGTTTCGCCAACCTAA: array number 23) and Primer X

(TTTTGTCTGACTAGACGCGTGAAGCATGCCC: array number 24). The Transporter gene field was amplified. It cut by NotI and Sall after checking this array, and permuted between NotI of pG3-N, and a Sall part. Next, UDP-GlcNAc which includes promoterregion by NaeI and SmaI from this plasmid The Transporter gene fragment was cut down and it inserted in the SmaI part of pYOM5. This plasmid was named pYOMR5. Said transformation of 52 shares of YCY(s) was performed by the acetic-acid lithium method using this plasmid. SD-Leu which contains 0.3M KCl after a transformation (it wound around the plate of the nucleobase except 2% glucose, 0.67% YeastNitrogen Base w/o amino acids (product made from Difco), and a leucine, and an amino acid mixture (20-400 mg/L) culture medium, and cultivated for two days at 30 degrees C, and the transformant was obtained.) This transformant was set to 73 shares of YCY(s). Enzyme activity is measured using the cell extract of 73 shares of YCY(s), and they are alpha-1, 2-mannosidase, and UDP-GlcNAc. The manifestation of Transporter was checked.

Moreover, the plasmid for carrying out integration of hUGTrel2 to msdS was produced. It is Sma about PGAMH. I, Nae It cuts by I, a msdS array is started with a GAP promotor, and it is Pvu of pRS404. It inserted in II part. This plasmid was named msdS-pRS404. It is Sma about the plasmid by which hUGTrel2 is inserted in a GAP promotor's lower stream of a river, and hUGTrel2-pG3. I, Nae It cuts by I, hUGThel2 array is started with a GAP promotor, and it is Pst of msdS-pRS404. It inserted in I part. This plasmid was named HM-pRS404. BstX in TRP1 of HM-pRS404 It cut by I and the transformation of 42 shares of YCY(s) was carried out using the acetic-acid lithium method. It is YPAD+0.3M [5ml] about a transformant. It checked that cultivated for two days at 30 degrees C, and msdS and hUGTrel2 were included in the chromosome of TRP1 by the PCR method by KCl. Moreover, enzyme activity was measured using the cell extract and the manifestation of the alpha-1, 2-mannosidase, and the UDP-GlcNAc transporter of both stocks was checked. The stock which carried out integration of hUGTrel2 to msdS at 42 shares of YCY(s) was set to 63 shares of TIY(s).

Furthermore, Homo sapiens liver alpha-mannosidase From vector pYEOM2-HA for a manifestation of II, the gene fragment which contains HA-tag by SacI and SphI is cut down, and it is DNA. T four The end was graduated by polymerase. This fragment was inserted in the SmaI part of pAUR123. Alpha-mannosidase which contains a promotor after checking being tied to the promotor in the direction of the right II gene field was started by BamHI and it inserted in the BamHI part of pRS406. This plasmid was made into the shape of a straight chain by NdeI, and the transformation of 63 shares of TIY(s) was performed by the acetic-acid lithium method. SD-Ura which contains 0.3M KCl after a transformation (it wound around the plate of 2% glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and an adenine, and an amino acid mixture (20-400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that Gnt-I and a Gnt-II gene were included in the chromosome of ADE2 field by the PCR method, and was referred to as 122 shares of YCY(s). Each enzyme activity was measured using the cell extract of 122 shares of YCY(s), and the manifestation of Gnt-I and Gnt-II was checked.

Next, the plasmid pRSGATP1 produced in the example 8 was made into the shape of a straight chain by NdeI, and the transformation of 122 shares of YCY(s) was performed by the acetic-acid lithium method. SD-His which contains 0.3M KCl after a transformation (it

wound around the plate of a glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and a histidine, and an amino acid mixture (20–400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that beta-1, 4-GalT, and UGT2 gene was included in the chromosome of HIS3 field by the PCR method, and was referred to as 142 shares of YCY(s). Each enzyme activity was measured using the cell extract of 142 shares of YCY(s), and the manifestation of beta-1, 4-GalT, and Ugt2p was checked. Furthermore, the transformation of 142 shares of YCY(s) was performed by the acetic-acid lithium method using the plasmid pYOMR5 shown in the example 8. SD-Leu which contains 0.3M KCl after a transformation (it wound around the plate of a glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and a leucine, and an amino acid mixture (20–400 mg/L) culture medium 0.67% 2%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) This transformant was set to 163 shares of YCY(s). Enzyme activity is measured using the cell extract of 163 shares of YCY(s), and they are alpha-1, 2-mannosidase, and UDP-GlcNAc. The manifestation of Transporter was checked.

About 163 shares of this YCY, in order to see change of the sugar chain structure of the mannan protein of the cell cortex of yeast, the lectin dye affinity was evaluated. Although it is known that concanavalin A will combine with the high mannose mold containing mannose 3 specific residue, a hybrid mold, a double strand compound-die sugar chain, etc., it is known that the high mannose mold sugar chain of the compatibility is more expensive compared with a hybrid mold and a double strand compound-die sugar chain. then, the yeast cell which carried out the harvest of the Texas-red indicator concanavalin A solution — mixing — sometimes — agitating — ** et al. — 4 degrees C was left for 2 hours. They are after washing and 10mM at PBS. Alpha-methyl It washed by PBS containing mannoside and observed under the fluorescence microscope. Consequently, in the YS134-4 A share, it checked that the fluorescence as which after washing was regarded around the cell by 163 shares of YCY(s) although fluorescent staining of the surroundings of a cell was carried out was decreasing clearly. By 163 shares of YCY(s), the sugar chains of a high mannose mold decreased in number from this, and it was suggested that the compound-die sugar chain is generating.

[Example 10] The production of the Homo sapiens fibroblast growth factor (FGF) in a yeast variant and the alteration of sugar chain structure which have mammals mold sugar chain productivity You supplied the FGF6-1 chimera gene (secFGF (N35)) from Ms. Atsuko Yoneda of National Institute of Bioscience and Human-Technology (Yoneda et al., BioTechniques, 27,576–590 (1999)). It is Sma about SecFGF (N35)/pBS. I, Nae It cut by I, FGF was started and it inserted in the HindIII part of pGEM2-alpha36. This plasmid was named pFGFalpha23. It is EcoR about pFGFalpha23. It cuts by I and is prepro. Alpha-factor and a FGF field are started and it is EcoR of pUC119 plasmid. It inserted in I part. This plasmid was named FGF-pUC119. In order to remove the EAEA array of alpha-factor, Primer Y (CGCCAGGGTTTTCCAGTCACGAC: array number 25) and Primer Z (ATGGGCGGCTCTTTTATCCAAAGATAC: array number 26) were used, and it amplified by PCR. It is EcoR of pUC18 about this DNA fragment. It included in I part and pAF02 plasmid was produced. It is Nae about pFGF01. I, Sma It cuts by I, FGF is started and it is Nae of pAF02. I, Sma It inserted in I part. This plasmid was named pAF03. It is EcoR about PAF03. It cuts by I and is prepro. Alpha-factor and a FGF field were started and the plasmid pAFF2 included in the lower stream of a river of the GAP promotor of a YEp352GAP plasmid was produced. It is Aat about pAFF2. II, Hpa It cut by I, 2-micrometer field was started, and the plasmid pAFF3 for yeast integration was built. Next, it is ApaI about pAFF3. I, Acc Pvu of the plasmid pYO325 which cuts by I, starts the array of FGF with a GAP promotor, and has LEU2 marker It inserted in II part. Furthermore, it is Spe about 2-micrometer field of a plasmid. It cut and removed by I. This plasmid was named pAFF9. EcoR in pAFF9 Linearization was cut and carried out by V and the transformation of the yeast (19 shares of TIY(s), 42 shares of YCY(s)) was carried out using the acetic-acid lithium method. It wound around the plate of a SD-Leu (2% glucose, excluding Yeast Nitrogen Base w/o amino acids (product made from Difco), and leucine 0.67% nucleobase, and amino acid mixture (20–400 mg/L)) culture medium after the transformation, and cultivated for two days at 30 degrees C, and the transformant was obtained, respectively.

It is YPAD+0.3M [5ml] about those transformants. It cultivated for three days at 30 degrees C, the heparin sepharose suspension (Pharmacia manufacture) of 50microl bed was added to culture supernatant liquid, it shook at 4 degrees C overnight, and FGF was made to stick to heparin sepharose by KCl. Then, heparin sepharose was collected in centrifugal and SDS-PAGE was presented with supernatant liquid after boiling by the SDS sample buffer. Western blotting was performed using the anti-FGF antibody, and it checked that FGF was discovered. Furthermore, it checked that FGF was included in the chromosome of LEU2 by the PCR method, and the stock which carried out integration of the FGF for the stock which carried out integration of the FGF to 19 shares of TIY(s) to 48 shares of TIY(s) and 42 shares of YCY(s) was set to 49 shares of TIY(s).

In order to perform a proteinic stable and efficient manifestation furthermore, the plasmid for carrying out integration of the msdS was produced. It is EcoR about the plasmid pGAMH by which msdS is inserted in a GAP promotor's lower stream of a river. It cut by I and the plasmid except 2-micrometer field was produced. This plasmid was named plmsdS. Xba in TRP1 of plmsdS It cuts by I. TIY48 (deltamnn1::hisG deltamnn4::hisG deltaoch1::hisG FGF::LEU2), TIY49 (deltamnn1::hisG deltamnn4::hisG deltaoch1::hisG FGF::LEU2 ade2::[GnT-I & GnT-II] his3::[beta-1.4-GalT & UGT2]) The transformation was carried out using the acetic-acid lithium method. It is YPAD+0.3M [5ml] about the obtained transformant. It cultivated for three days at 30 degrees C, the heparin sepharose (Pharmacia manufacture) of 50microl was added to culture medium, it shook at 4 degrees C overnight, and FGF was made to stick to heparin sepharose by KCl. Then, heparin sepharose was collected, Western blotting was performed by the antibody of FGF, and it checked that msdS was discovered. Furthermore, it checked that msdS was included in the chromosome of TRP1 by the PCR method. The stock which carried out integration of the msdS for the stock which carried out integration of the msdS to 48 shares of TIY(s) to 53 shares of TIY(s) and 49 shares of YCY(s) was set to 54 shares of TIY(s).

Next, the plasmid for carrying out integration of hUGTrel2 to msdS was produced. It is Sma about PGAMH. I, Nae It cuts by I, a msdS array is started with a GAP promotor, and it is Pvu of pRS404. It inserted in II part. This plasmid was named msdS-pRS404. It is Sma about the plasmid by which hUGTrel2 is inserted in a GAP promotor's lower stream of a river, and hUGTrel2-pG3. I, Nae It cuts by I, hUGTrel2 array is started with a GAP promotor, and it is Pst of msdS-pRS404. It inserted in I part. This plasmid was named HM-pRS404. BstX in TRP1 of HM-pRS404 It cut by I and the transformation of 48 shares of TIY(s) and 49 shares of YCY(s) was carried out using the acetic-acid lithium method. It is YPAD+0.3M [5ml] about a transformant. It cultivated for three days at 30 degrees C, the heparin sepharose suspension (Pharmacia manufacture) of 50microl bed was added to culture supernatant liquid, it shook at 4 degrees C overnight, and FGF was made to stick to heparin sepharose by KCl. Then, heparin sepharose was collected in centrifugal and SDS-PAGE was presented with supernatant liquid after boiling by the SDS sample buffer. Western blotting was performed using the anti-FGF antibody, and it checked that FGF was discovered. Furthermore, it checked that msdS and hUGTrel2 were included in the chromosome of TRP1 by the PCR method. Moreover, enzyme activity was measured using the cell extract and the manifestation of the alpha-1, 2-

mannosidase, and the UDP-GlcNAc transporter of both stocks was checked. The stock which carried out integration of hUGrrel2 for the stock which carried out integration of msdS and hUGTrel2 to 48 shares of TIY(s) to msdS at 59 shares of TIY(s) and 49 shares of TIY(s) was set to 60 shares of TIY(s).

For preparation of a sugar chain, 48 shares of TIY(s) and 53 shares of TIY(s) which carried out integration of the FGF on the chromosome of LEU2 were used, and FGF was refined from the culture medium of 3L. YPAD+0.3M of 3L After culture, centrifugal was carried out, 2ml heparin sepharose was added to the culture medium except a cell, it shook at 4 degrees C overnight, and FGF was made to stick to heparin sepharose for 30 degrees C and three days by KCl. Heparin sepharose is collected and they are a click and PBS+0.01% to a column. CHAPS, PBS+2.5M NaCl+0.01% FGF was made eluted from heparin sepharose by lifting of salt concentration by using CHAPS as a solvent.

It desalted by applying 150micro of refined FGF abbreviation g to an opposite phase column. A column is muRPC. C2/C18 PC3.2/3 column (Pharmacia manufacture) is used, and it is 0.1%. Trifluoroacetic acid and 0.1% -60% of trifluoroacetic acid Elution from an opposite phase column was performed by using an acetonitrile as a solvent.

The sample eluted from the column was dried and hydrazinolysis was performed. The 2ml hydrazine was added by the vacua and it processed for 60 minutes at 110 degrees C. Then, it cooled to the room temperature and N-acetylation was performed. it was easy to add the 0.2M ammonium acetate of 250microl, and the acetic anhydride of 25microl, they were agitated, and it was left at the room temperature for 30 minutes. furthermore, it was easy to add the 0.2M ammonium acetate of 250microl, and the acetic anhydride of 25microl, they were agitated, and it was left at the room temperature for 30 minutes. Concentration hardening by drying of the reaction mixture was carried out, and it considered as the sugar chain adjustment article.

The following actuation was performed in order to carry out fluorescent labeling (pyridylamino-izing) of the obtained sugar chain. After concentration hardening by drying, the coupling reagent (300mg 2-aminopyridine was dissolved in the acetic acid of 100microl) of 20microl was added and sealed, and 90 degrees C of sugar chain adjustment articles were processed for 60 minutes. Then, the reduction reagent (10mg borane dimethylamine complex was dissolved in the acetic acid of 50microl) of 20microl was added and sealed, and was processed for 60 minutes 80 degrees C. After the reaction, after carrying out addition candle power churning of the triethylamine-methanol of 20microl, it was easy to add the toluene of 40 moremicrol, it was agitated, and 60 degrees C carried out concentration-under nitrogen air current hardening by drying for 10 minutes. Then, after being easy to add the methanol of 20microl and agitating it to reaction mixture, it was easy to add the toluene of 40microl, it was agitated, and 60 degrees C carried out concentration-under nitrogen air current hardening by drying for 10 minutes. This was repeated 3 times, the toluene of 50microl was added to residue, and 60 degrees C carried out concentration-under nitrogen air current hardening by drying for 10 minutes. HW-40 gel-filtration column processing was performed after the reaction, and unreacted 2-aminopyridine was removed.

HPLC using an amino column performed sugar chain structural analysis. A column is Asahipak. Using NH2P-50 (4.6mmx250mm), the solvent adjusted the mixed liquor (A liquid) of 7:3 of the 200mM acetic-acid-triethylamine buffer solution (pH7.3) and an acetonitrile, and the mixed liquor (B liquid) of 2:8 of the 200mM acetic-acid-triethylamine buffer solution (pH7.3) and an acetonitrile.

By pouring Solvent A by rate-of-flow 1.0 ml/min beforehand, the column was equilibrated, the rate of Solvent B was linearly raised from immediately after sample impregnation to 100% over 50 minutes, and the sink and PA-ized oligosaccharide were eluted for 20 minutes in the rate of Solvent B after that with 100%. The analysis result was shown in [drawing 14](#) . In the thing of the TIY48 origin, like the result of an example 2, it is mainly one peak ([drawing 14](#) , upper case), and was in agreement with the elution location of a Man8GlcNAc2-PA preparation (TAKARA SHUZO make). On the other hand, in 53 shares of TIY(s) containing alpha-1 and a 2-mannosidase gene, one peak was mainly seen ([drawing 14](#) , lower berth). The Man5GlcNAc2-PA preparation and the elution location of this peak corresponded.

Therefore, FGF which is the Homo sapiens glycoprotein made to discover by 53 shares of TIY(s) became clear [having the Man5GlcNAc2 mold sugar chain which is the precursor of an about 100% hybrid mold and a compound die].

Furthermore, Homo sapiens liver alpha-mannosidase From vector pYEOM2-HA for a manifestation of II, the gene fragment which contains HA-tag by SacI and SphI is cut down, and it is DNA. T four The end was graduated by polymerase. This fragment was inserted in the SmaI part of pAUR123. Alpha-mannosidase which contains a promotor after checking being tied to the promotor in the direction of the right II gene field was started by BamHI and it inserted in the BamHI part of pRS406. This plasmid was made into the shape of a straight chain by NdeI, and the transformation of 60 shares of TIY(s) was performed by the acetic-acid lithium method. SD-Ura which contains 0.3M KCl after a transformation (it wound around the plate of 2% glucose, the nucleobase except Yeast Nitrogen Base w/o amino acids (product made from Difco), and a uracil, and an amino acid mixture (20-400 mg/L) culture medium 0.67%, and cultivated for two days at 30 degrees C, and the transformant was obtained.) From the transformant, genomic DNA was prepared, and it checked that the gene was included in the chromosome of URA3 field by the PCR method, and was referred to as one share of MSY. Enzyme activity is measured using the cell extract of one share of MSY, and it is alpha-mannosidase. The manifestation of II was checked.

Availability on industry According to the auxotroph Mie variant and four-fold auxotroph variant which carried out the breeding newly by this invention, the glycoprotein which has the same neutral sugar chain as the high mannose mold which mammals cells, such as Homo sapiens, produce, or the same neutral sugar chain is producible with a large quantity and sufficient purity. Moreover, the protein which has mammals mold sugar chains, such as a high mannose mold, a hybrid mold, and a compound die, or a mammals mold sugar chain is efficiently producible by introducing the biosynthesis system gene of a mammals mold sugar chain into the variant concerned.

[Layout Table]

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Secretary of Agency of Industrial Science and Technology

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[Brief Description of the Drawings]

Drawing 1 shows the biosynthetic path of the common N-joint mold sugar chain in a mammals animal.

Drawing 2 shows the biosynthetic path of the N-joint mold sugar chain in yeast (*S.cerevisiae*). H, C, and E follow I, D, and F of drawing 3 among drawing, respectively.

Drawing 3 shows the biosynthetic path (continuation) of the N-joint mold sugar chain in yeast (*S.cerevisiae*).

Drawing 4 shows the destructive procedure of the conventional yeast gene.

Drawing 5 shows how to destroy a gene, without introducing eventually the gene which carries out the complementation of the auxotroph.

Drawing 6 shows structural analysis of a 19 shares of TIY(s) cell-cortex mannan protein sugar chain.

Drawing 7 shows structural analysis in Amide-80 column of the cell-cortex mannan protein sugar chain of 19 shares of TIY(s) which introduced alpha-1 and a 2-mannosidase gene.

a: Sugar chain b:alpha -1 of the mannan glycoprotein of 19 shares of TIY(s), sugar chain of the mannan glycoprotein of 19 shares of TIY(s) which introduced 2-mannosidase Drawing 8 shows structural analysis in the ODS-80TM column of the cell-cortex mannan protein sugar chain of 19 shares of TIY(s) which introduced alpha-1 and a 2-mannosidase gene.

a: The standard sugar chain b of the structure shown by the formula (III) : fraction isolated preparatively by drawing 6 Drawing 9 shows the result of GnT-I activity measurement.

Drawing 10 shows structural analysis in Amide-80 column of the cell-cortex mannan protein sugar chain of 19 shares of TIY(s) which introduced alpha-1, the 2-mannosidase gene, and the GnT-I gene.

A: Only a vector Sugar chain structural-analysis [of 19 shares of introduced TIY(s)] B: Sugar chain structural-analysis

a:Man5GlcNAc2-PAb:GlcNAcMan5GlcNAc2-PAc:Man6GlcNAc2-PAd of 19 shares of TIY(s) which introduced alpha-1, the 2-mannosidase gene, and GnT-1 gene : Man7GlcNAc2-PAe:Man8GlcNAc2-PA Drawing 11 shows structural analysis in the ODS-80TM column of the cell-cortex mannan protein sugar chain of 19 shares of TIY(s) which introduced alpha-1, the 2-mannosidase gene, and the GnT-I gene.

A: Mixture B of a reference standard : fraction isolated preparatively by drawing 10 .B Drawing 12 is alpha-mannosidase. The western blot analysis using the cell extract of 500 shares of YPH(s) which introduced II gene is shown.

A: The western-blot-analysis result B of the cell extract from 500 shares of YPH(s) into which only the vector introduced (pYEX-BX-3HA) : western-blot-analysis result of the cell extract of 500 shares of YPH(s) which introduced the vector (pYEOM2-HA) containing a chimera alpha-mannosidase-II gene Drawing 13 is alpha-mannosidase. Alpha-mannosidase using the cell extract of 500 shares of YPH(s) which introduced II gene The result of II activity measurement is shown.

Only a vector A: (pYEX-BX-3HA) Activity measurement YPH(s) [which were introduced / 500 shares of] result B: YPH(s) [which introduced the vector (pYEOM2-HA) containing a chimera alpha-mannosidase-II gene / 500 shares of] activity measurement result

a:GlcNAcMan5GlcNAc2-PAb:GlcNAcMan3GlcNAc2-PA Drawing 14 Structural analysis in Amido-80 column of the FGF sugar chain of 53 shares (lower berth) of TIY(s) which introduced 48 shares (upper case) of TIY(s) which introduced the FGF gene and the FGF gene, and alpha-1 and a 2-mannosidase gene is shown.

Explanation GlcNAc and GN of a sign: N-acetyl glucosamines Man and M : Mannose PA : Formation of 2-amino pyridyl

[Translation done.]

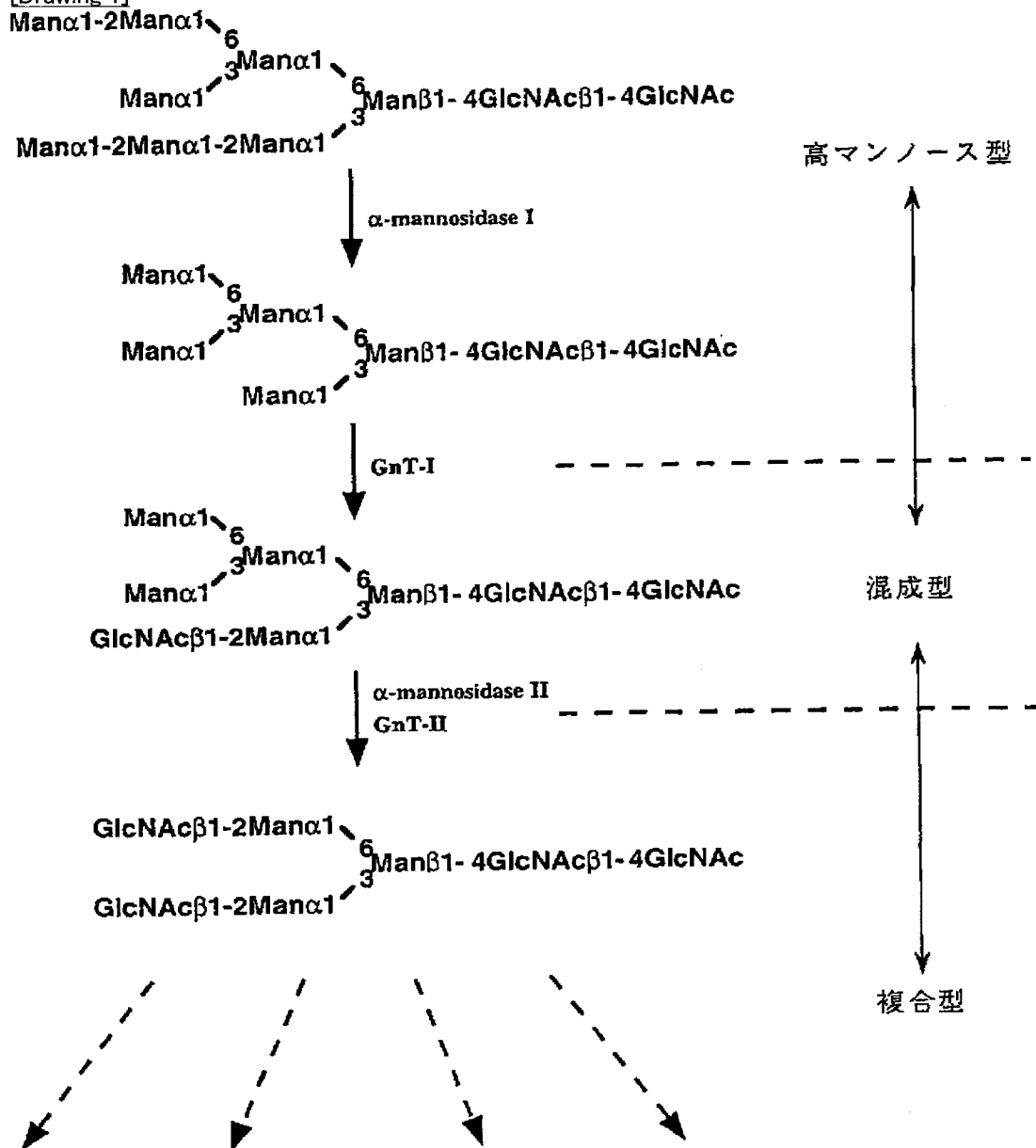
* NOTICES *

JPO and INPIT are not responsible for any damages caused by the use of this translation.

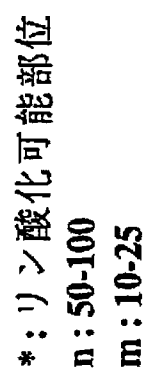
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- 2.**** shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

DRAWINGS

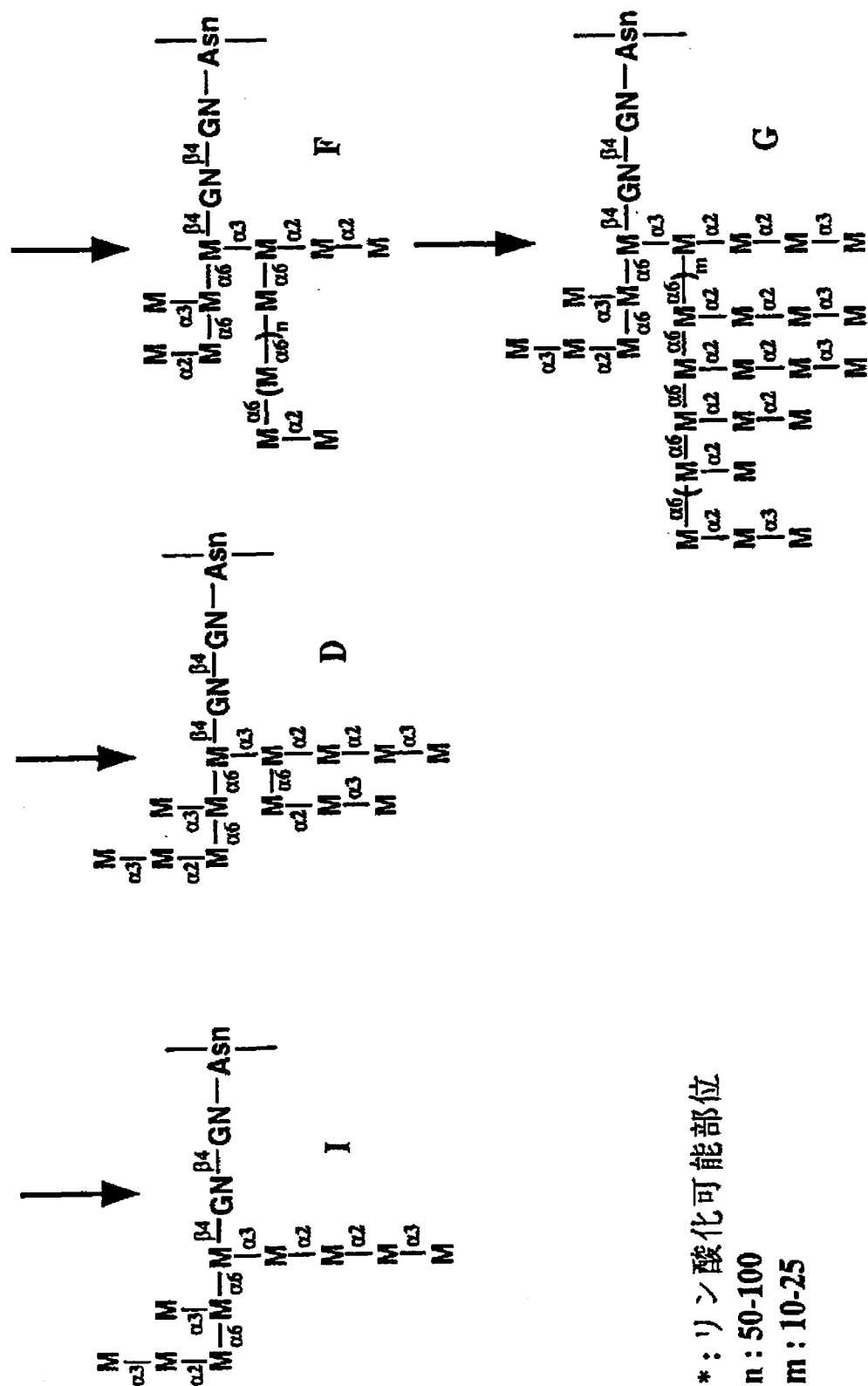
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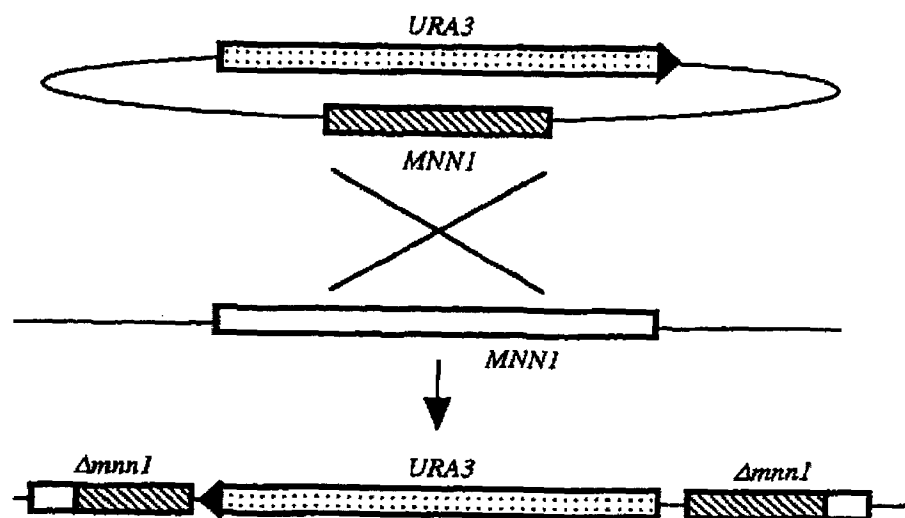
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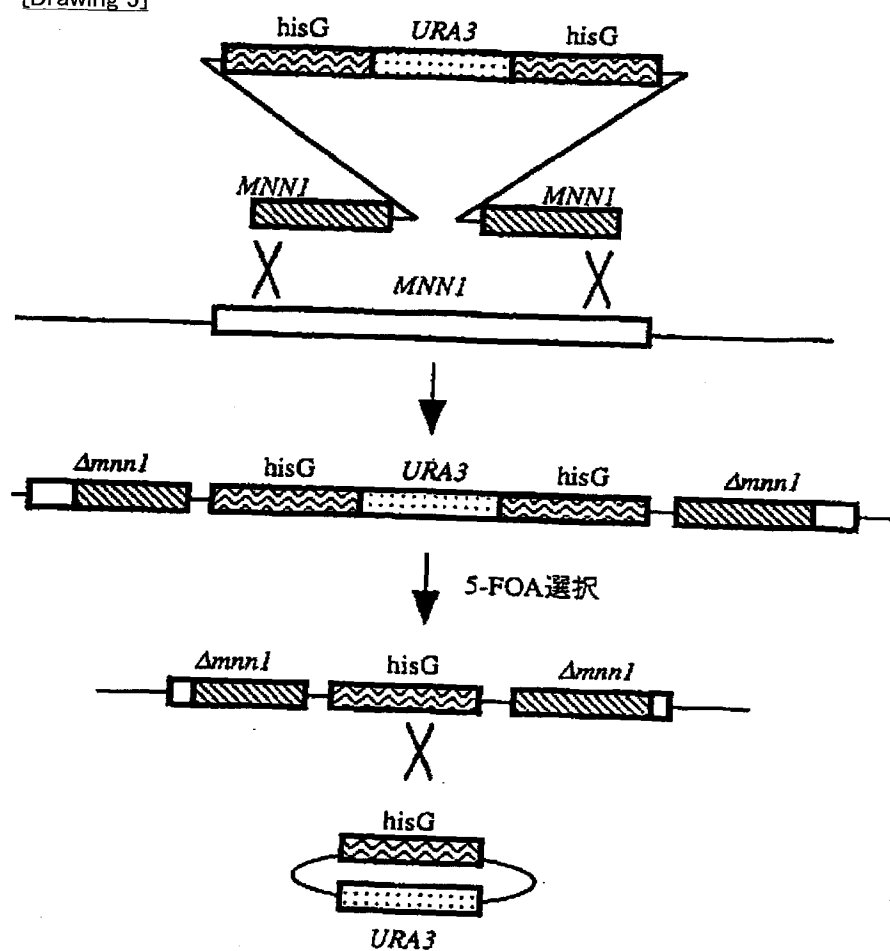
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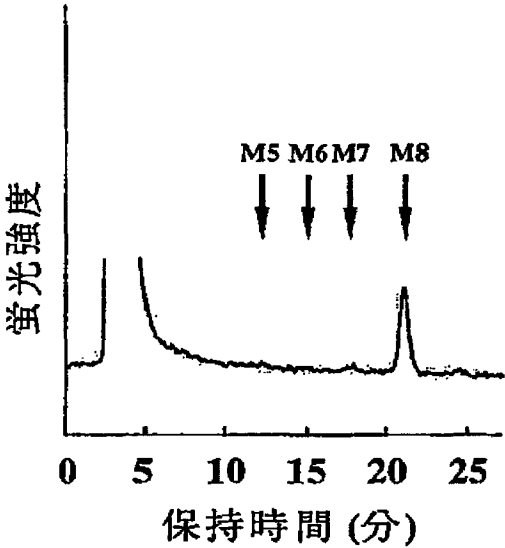
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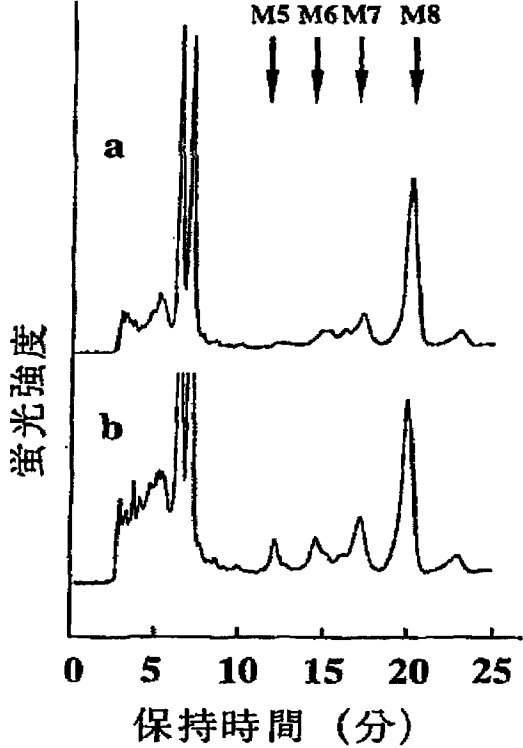
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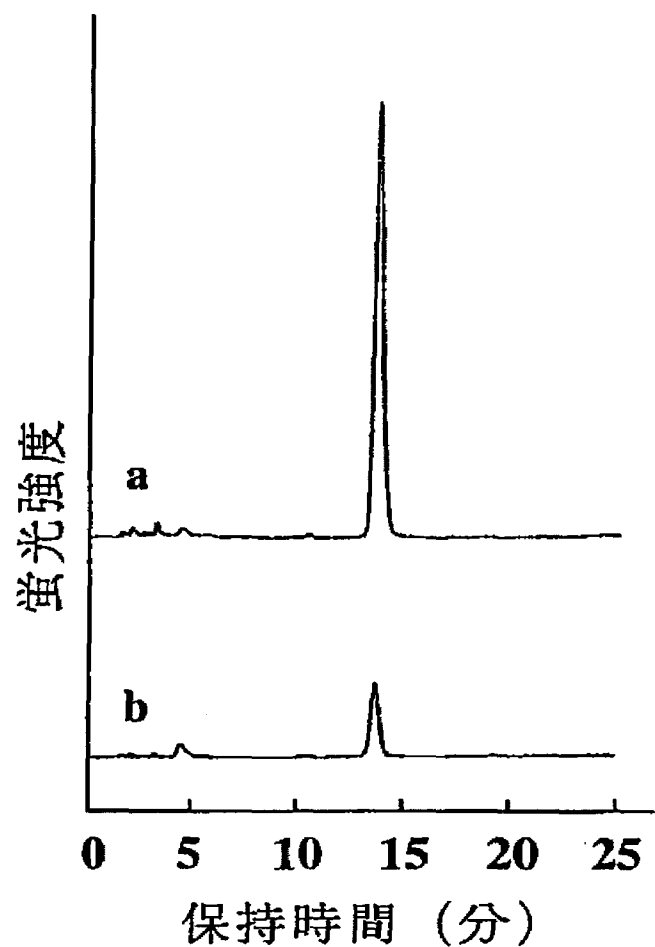
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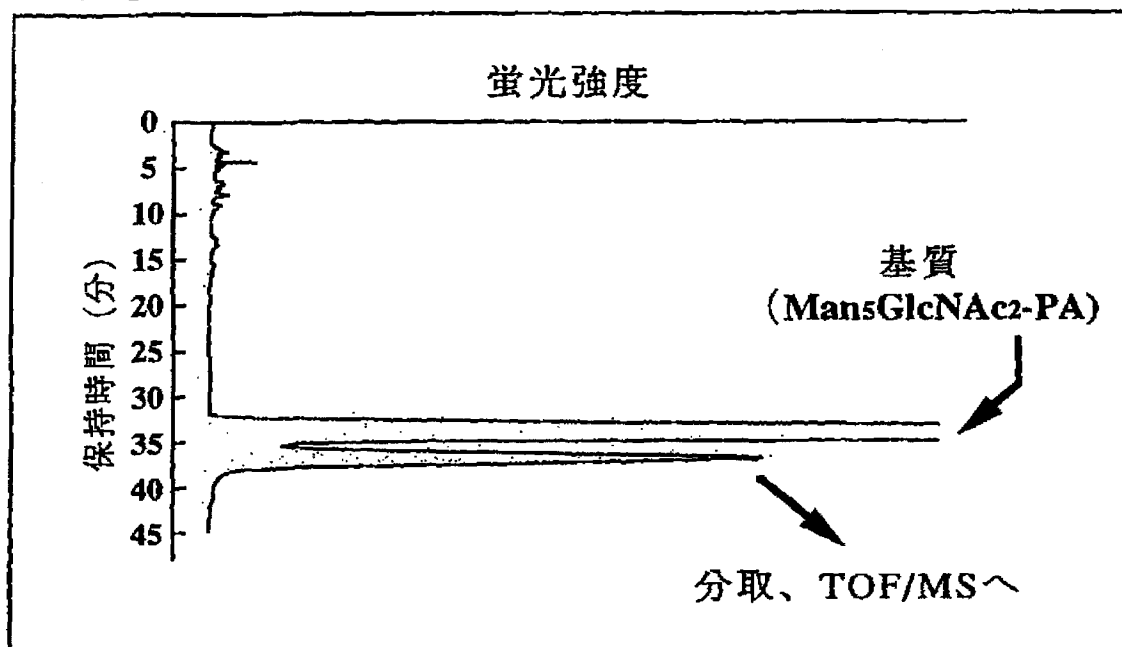
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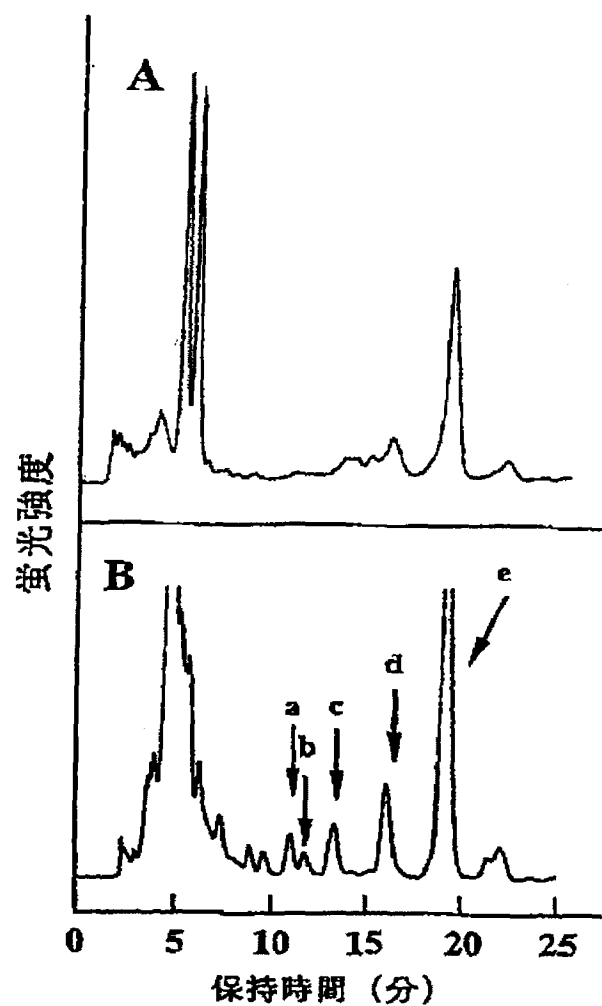
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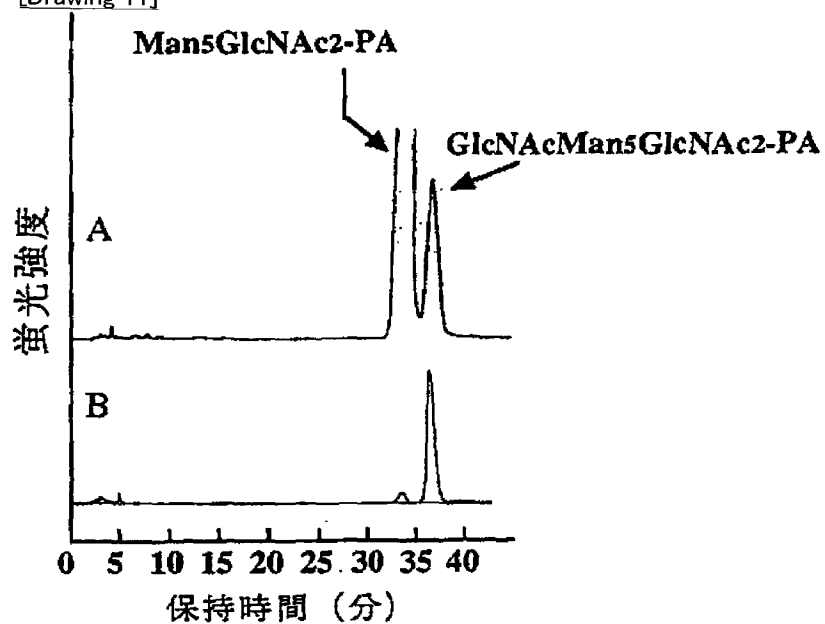
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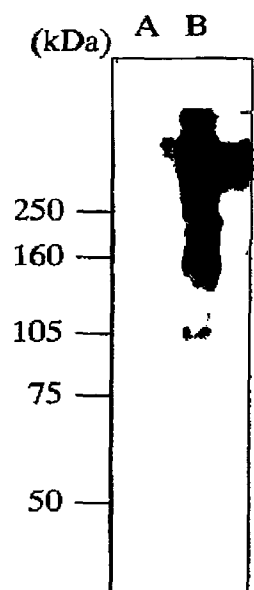
[Drawing 10]



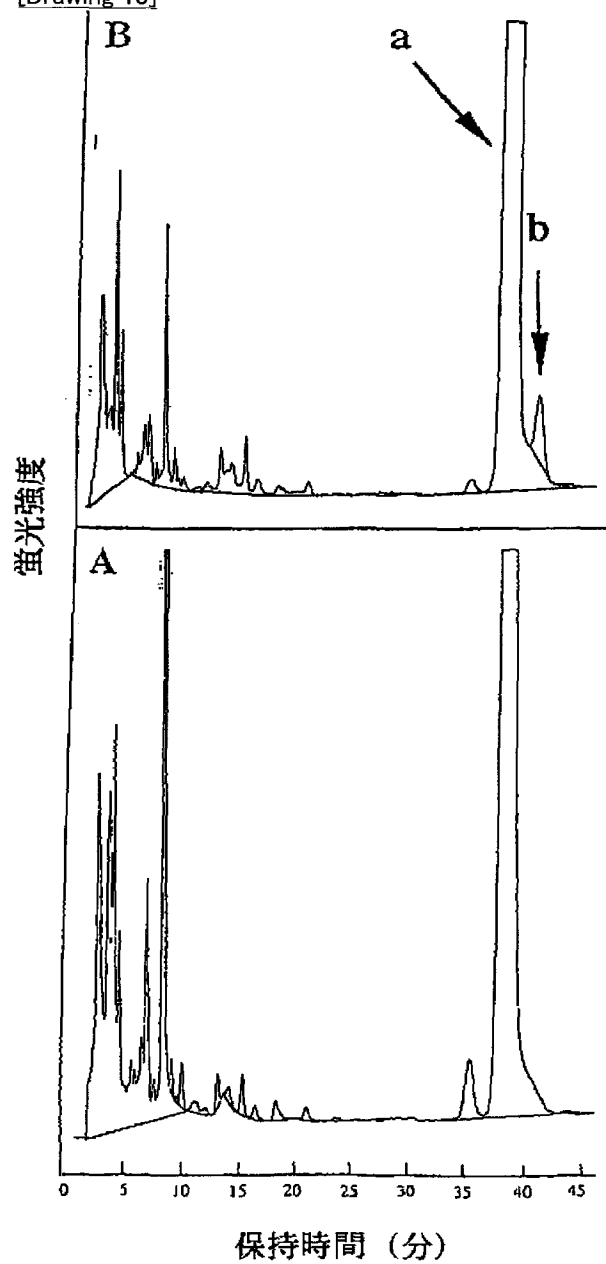
[Drawing 11]



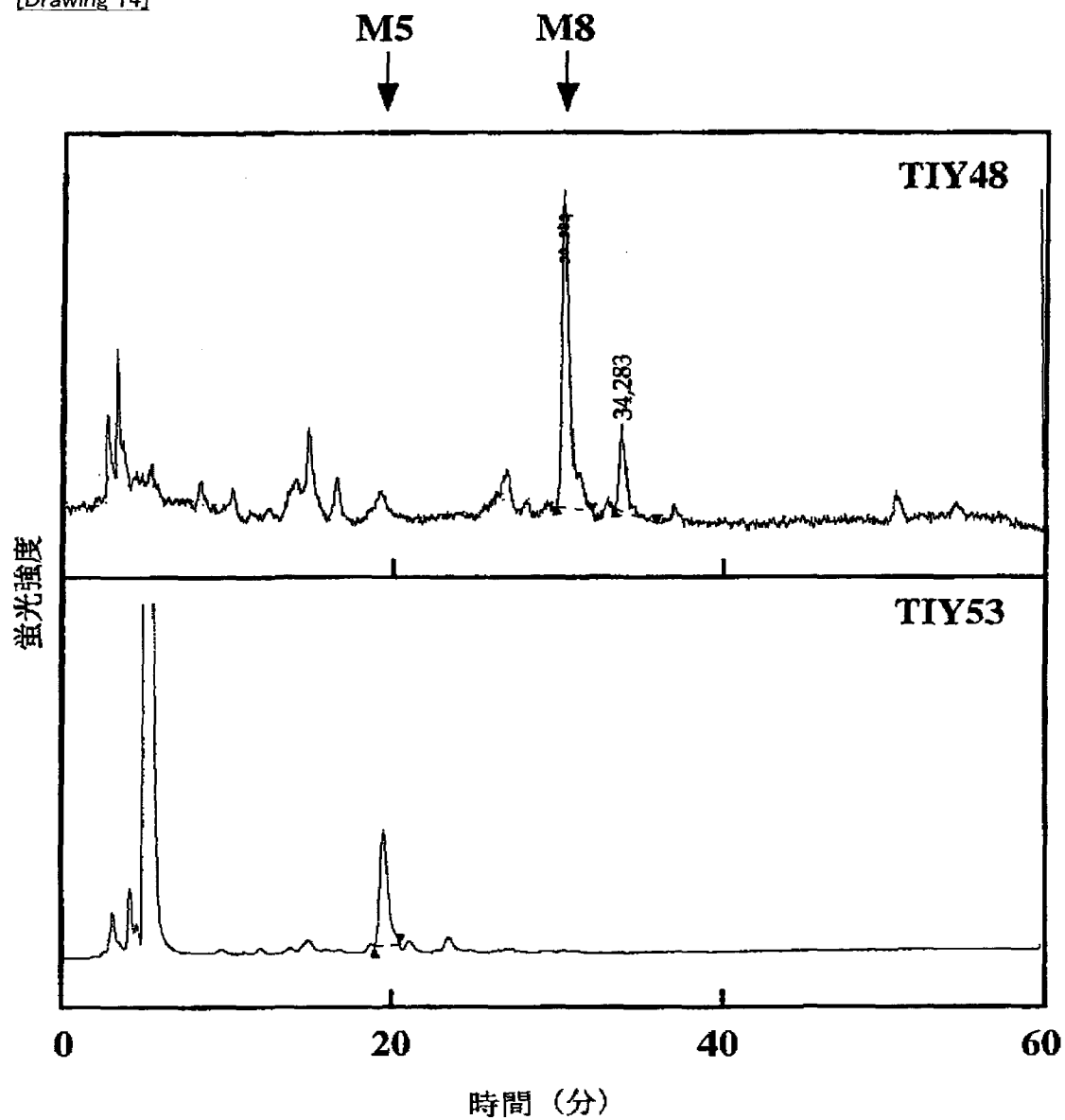
[Drawing 12]



[Drawing 13]



[Drawing 14]



[Translation done.]



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<p>(54) Title: YEAST STRAINS</p> <p>(57) Abstract</p> <p>Reduction (preferably elimination) of the HSP150 protein in a yeast used to produce desired foreign proteins, especially human albumin, facilitates subsequent purification of the protein.</p> <div data-bbox="893 1113 1510 1932"> <p>5' and 3' regions of HSP150 gene obtained by PCR:</p> <pre> graph TD subgraph 5_prime [5' region] A[EcoR I] --> B[5'] B --> C[NotI] end subgraph 3_prime [3' region] D[NotI] --> E[3'] E --> F[EcoR I] end A --> G[Cloned into pUC19] C --> G D --> H[Cloned into pUC19] F --> H G --> I((pAYE503)) I --> J[Sequenced] J --> K[EcoR I/Hind III fragment isolated] H --> L((pAYE504)) L --> M[Sequenced] M --> N[EcoR I/Hind III fragment isolated] K --> O[] N --> O O --> P[] </pre> </div>		

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YEAST STRAINS

Field of the Invention

- 5 The present invention relates to the production of heterologous proteins by yeast species and more particularly to an adaptation of the yeast in which the protein is produced.

Background and Prior Art

10

- In recent years, yeasts have been widely used as host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992), including recombinant human albumin (rHA) (Sleep *et al*, 1990, 1991; Fleeer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be
15 grown to high cell density on simple media, and as eukaryotes are suitable for the production of secreted as well as cytosolic proteins.

- When yeasts are utilised to produce a desired heterologous protein by secretion into the growth medium, a large number of host-derived proteins may also be
20 present, including other proteins secreted by the host but also intracellular proteins present in the supernatant as the result of leakage from cells or cell lysis. In processes in which the protein is not secreted, there is of course an even higher level of contamination with intracellular yeast proteins. It is necessary to purify the desired protein and to remove these contaminating
25 proteins from the preparation; such methods have been disclosed in WO 92/04367 and EP 524 681. The majority of contaminating proteins will have physicochemical properties sufficiently different from the desired protein to permit efficient separation by standard techniques, such as ion exchange or size exclusion chromatography. The prior art gives the impression that such
30 proteins can be satisfactorily removed by such techniques; see, for example EP

524 681 (Gist-brocades), EP 570 916 (Green Cross) and EP 464 590 (Green Cross). Indeed, we have developed sophisticated chromatographic techniques (unpublished) to remove contaminating proteins from desired proteins.

5 Summary of the Invention

We have now also adopted a different approach and have identified the gene responsible for a protein, namely the *HSP150* gene, which co-purifies with recombinant human albumin (rHA) and, in principle, with other desired
10 proteins. In accordance with the invention, we eliminate the contaminating protein from the initial fermentation, rather than develop highly sophisticated and complex means of removal during purification. This protein was not previously known to be a co-purifying contaminant.

15 In one aspect of the invention, the *HSP150* gene is functionally deleted from the genome of the host. This has not caused any detrimental effects on production of the desired protein and removes a potential contaminant that has proven difficult to remove by standard purification techniques. Despite the presence of at least two closely related genes encoding proteins very similar to
20 Hsp150, *PIR1* and *PIR3*, in such modified yeast, rHA purified from these organisms does not contain detectable levels of any protein from this family.

The *S. cerevisiae* Hsp150 protein was originally described by Russo *et al* (1992) and was shown to be produced constitutively, to be extensively O-
25 glycosylated and to be secreted efficiently into the growth medium. A 7-fold increase in the level of Hsp150 protein was seen when cells grown at 28°C were shifted to 37°C. Makarow has proposed preparing fusions of Hsp150 (or fragments thereof) and a desired protein, in order to achieve enhanced, controllable secretion (WO 93/18167). The *HSP150* gene encodes a primary
30 translation product of 413 amino acids, including an N-terminal secretion signal

sequence of 18 amino acids that is not present in the mature protein. A further post-translational processing event occurs C-terminal to a pair of basic residues to yield two subunits of 54 and 341 amino acids which remain associated. The 341 amino acid subunit contains 11 tandem repeats of a 19 amino acid sequence, the function of which is unknown. Homologues of the *HSP150* gene were found in *Torulaspora delbrueckii*, *Kluyveromyces marxianus* and *Schizosaccharomyces pombe* (Russo *et al*, 1992).

The same protein has been designated the PIR2 protein by Toh-e *et al* (1993). The *HSP150/PIR2* gene was shown to be a member of a family of at least three genes (*PIR1*, *PIR2* and *PIR3*) all of which contain similar internal tandem repeats of approximately 19 amino acids. Homologues of the *PIR* genes were shown to be present also in *Kluyveromyces lactis* and *Zygosaccharomyces rouxii* (Toh-e *et al*, 1993). Disruption of the *HSP150/PIR2* gene showed that this is not an essential gene (Russo *et al*, 1992; Toh-e *et al*, 1993).

In this specification we refer to rHA as the desired protein. However, it is to be understood that the problem addressed by the invention will, in principle, be encountered with any other protein which has similar properties to those of rHA and which is therefore purified in the same way. Thus, the solution provided by the invention, namely elimination of Hsp150, is applicable also to the production of such other proteins.

Our studies have revealed that the Hsp150 protein is inefficiently separated from rHA by ion exchange chromatography. Surprisingly, however, Hsp150 does not appear in the fraction equivalent to the rHA fraction when rHA is absent. For example, when rHA-containing culture supernatant is passed through a cation exchange column under conditions which ensure binding of the rHA to the column (eg pH4.5, conductivity <7mS), Hsp150 also binds to the column and is eluted under the same conditions as rHA and thus contaminates

the rHA preparation. However, when culture supernatant from a yeast that does not secrete rHA is passed through such a column under the same conditions, the Hsp150 protein does not bind to the matrix but passes straight through the column. The eluate fraction does not contain Hsp150 in the
5 absence of rHA. Similarly, the Hsp150 protein does not bind to an anion exchange column run under conditions which would result in binding of albumin (eg pH5.5, 1.5mS) in the absence of rHA, but is present in the rHA eluate fraction when rHA is present. Surprisingly, we have found that the presence of rHA in culture supernatant significantly alters the behaviour of
10 some yeast proteins during chromatographic purification of the rHA such that proteins with physico-chemical properties which indicate that they would be separated from albumin by, for instance, ion exchange chromatography in fact contaminate the rHA preparation and are difficult to remove.

15 One aspect of the invention provides a process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).

The most convenient way of achieving this is to create a yeast which has a
20 defect in its genome such that a reduced level of the Hsp150 protein is produced. For example, there may be a deletion, insertion or transposition in the coding sequence or the regulatory regions (or in another gene regulating the expression of the Hsp150 gene) such that little or no Hsp150 protein is produced. Alternatively, the yeast could be transformed to produce an anti-
25 Hsp150 agent, such as an anti-Hsp150 antibody.

To modify the *HSP150* gene so that a reduced level of co-purifying protein is produced, site-directed mutagenesis or other known techniques can be employed to create single or multiple mutations, such as replacements, insertions,
30 deletions, and transpositions, as described in Botstein and Shortle, "Strategies

and Applications of *In Vitro* Mutagenesis", *Science*, 229: 193-210 (1985), which is incorporated herein by reference. Suitable mutations include chain termination mutations (clearly stop codons introduced near the 3' end might have insufficient effect on the gene product to be of benefit; the person skilled
5 in the art will readily be able to create a mutation in, say, the 5' three quarters of the coding sequence), point mutations that alter the reading frame, small to large deletions of coding sequence, mutations in the promoter or terminator that affect gene expression and mutations that de-stabilize the mRNA. Some desirable point mutations or specific amino acid substitutions may affect
10 chromatographic behaviour by altering the charge distribution. Hence, the protein produced has a similar primary amino acid sequence to that of native Hsp150, but is functionally distinct such that it will not co-purify with the desired protein. Such a modified protein is not regarded as being Hsp150. Specific mutations can be introduced by an extension of the gene disruption
15 technique known as gene transplacement (Winston, F. *et al* (1983) *Methods Enzymol.* 101, 211-228).

Any polypeptides inserted into the Hsp150 protein should not be, and should not create, ligands for the desired protein. Those skilled in the art can readily
20 determine, by simple binding assays, whether a ligand has been used or created. Generally one uses a selectable marker to disrupt a gene sequence, but this need not be the case, particularly if one can detect the disruption event phenotypically. In many instances the insertion of the intervening sequence will be such that a stop codon is present in frame with the Hsp150 sequence and the
25 inserted coding sequence is not translated. Alternatively the inserted sequence may be in a different reading frame to Hsp150.

The gene may have one or more portions (optionally including regulatory regions, up to the whole gene) excised or inverted, or it may have a portion
30 inserted, in order to result either in no production of protein from the *HSP150*

locus or in the production of protein from the *HSP150* locus which does not co-purify with the desired protein.

Preferably, the yeast secretes the desired protein, which is then purified from
5 the fermentation medium. The purification may take place elsewhere; hence, production of culture medium, containing desired protein, in which the level of Hsp150 protein is low or zero is an end in itself.

A protein is generally regarded as co-purifying with Hsp150 if the two are still
10 associated after two dissimilar chromatographic separation techniques (one of which is affinity chromatography for the desired protein) or, if affinity chromatography is not used, if the proteins are still associated after three dissimilar steps (for example an anion exchange, a cation exchange and a gel permeation step). Essentially, the identity of the desired protein is self-defined:
15 if a person skilled in the art finds that his desired protein is, after an otherwise suitable purification process, contaminated with a yeast protein, he can determine (using known methods, which are explained in more detail below) whether that yeast protein is Hsp150 and, if it is, use the yeasts and methods of the invention; if the desired protein is not contaminated with Hsp150, then
20 the need for the present invention will not arise. We have found the process of the invention to be particularly applicable to albumins and to other proteins which have similar physico-chemical properties to albumins, such that they are purified by similar chromatographic techniques. Preferably, the desired protein is a human albumin.

25

Human serum albumin (HSA) is a protein of 585 amino acids that is present in human serum at a concentration of 35-45g L⁻¹ and represents about 60% of the total serum protein. HSA is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and
30 exogenous ligands. It is used clinically in the treatment of patients with severe

burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

- 5 The albumin may be a variant of normal HSA/rHA. By "variants" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human
10 albumin, for example those fragments disclosed in EP 322 094 (namely HSA (1-n), where n is 369 to 419); and fusions of albumin with other proteins, for example the kind disclosed in WO 90/13653.

By "conservative substitutions" is intended swaps within groups such as Gly,
15 Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

A second main aspect of the invention provides a yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in such Hsp150.

20

In addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

25

The desired protein is produced in conventional ways, for example from a coding sequence inserted in the yeast chromosome or on a free plasmid.

The yeasts are transformed with a coding sequence for the desired protein in
30 any of the usual ways, for example electroporation. Methods for

transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

5 Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct can be grown to produce the desired polypeptide. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985)
10 *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies.

Useful yeast plasmid vectors include pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037,
15 USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

20 A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

25 Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that
30 remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic

activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar
5 excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then
10 cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International
15 Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA in accordance with the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491. In this method the DNA to be enzymatically amplified is flanked by
20 two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

25 Any yeast which produces an Hsp150 protein can be modified in accordance with the invention. Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia* (*Hansenula*), *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*,
30 *Leucosporidium*, *Botryosascus*, *Sporidiobolus*, *Endomycopsis*, and the like.

- Preferred genera are those selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, and *Torulaspora*. Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* spp. are *K. fragilis*, *K. lactis* and *K. marxianus*.
- 5 A suitable *Torulaspora* species is *T. delbrueckii*. Examples of *Pichia* (*Hansenula*) spp. are *P. angusta* (formerly *H. polymorpha*), *P. anomala* (formerly *H. anomala*) and *P. pastoris*.

- Homologues of *HSP150* have already been shown to be present in a wide range
- 10 of different yeast genera: *Torulaspora* sp., *Kluyveromyces* sp., *Schizosaccharomyces* sp. and *Zygosaccharomyces* sp. (Russo *et al*, 1992; Toh-*et al*, 1993). In addition, our own studies have shown by Southern blotting that *Pichia* sp. possess a homologue of *HSP150*.

- 15 Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

- Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes
- 20 for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, the *GPD1*
- 25 promoter, and hybrid promoters involving hybrids of parts of 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

- Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are
- 30 the thiamine-repressible promoter from the *nmr* gene as described by Maundrell

(1990) *J. Biol. Chem.* **265**, 10857-10864 and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990) *Genetics* **124**, 807-816.

- Methods of transforming *Pichia* for expression of foreign genes are taught in,
5 for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.
- 10 The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being
15 *PGK1*.

- The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be
20 those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

- The desired protein may be initially expressed with a secretion leader sequence,
25 which may be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae* include that from the mating factor α polypeptide (MF α -1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. Further such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed
30 in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-

sequence of MF α -1, β -glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α -galactosidase (*MELI*); *K. lactis* killer toxin; and *Candida* glucoamylase.

5 Detailed Description of the Invention

Preferred aspects of the invention will now be described in more detail, with reference to the accompanying drawings, in which

- 10 Figure 1 is a scheme showing the preparation of an *EcoRI* *HSP150-URA3-HSP150* fragment used to transform a yeast strain (DBU3) and disrupt the *HSP150* gene (Example 1); and

- 15 Figure 2 is a scheme showing the preparation of a further *EcoRI* fragment used to remove the *HSP150* coding sequence altogether (Example 2).

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding rHA are derived from the cDNA disclosed in EP 201 239.

20

Example 1

- 25 The *HSP150* gene was mutated by the process of gene disruption (Rothstein, 1983) which effectively deleted part of the *HSP150* coding sequence, thereby preventing the production of Hsp150.

Four oligonucleotides suitable for the PCR amplification of the 5' and 3' ends of the *HSP150* gene (Russo *et al*, 1992) were synthesized using an Applied Biosystems 380B Oligonucleotide Synthesizer.

30

5' End

LRE45: 5'-CTATTCCTATTTCTGGGAATTCTTAAAGACAAAAAGCTC-3'

LRE46: 5'-GGCTGTGGTGCTGCAGATGATGCGCTGG-3'

5 3' End

LRE47: 5'-GCTACTTCCGCTTCTGCAGCCGCTACCTCC-3'

LRE48: 5'-GCCGTGTAGCGAGGGAATTCTGTGGTCACGATCACTCG-3'

Note, LRE45 and LRE48 contain changes in the *HSP150* gene sequence so as to introduce *EcoRI* sites into the 5' or the 3' end of the *HSP150* gene PCR products. LRE46 and LRE47 both contain *Pst* I sites naturally present in the *HSP150* gene sequence (SEQ 1).

PCR was carried out to amplify individually the 5' and 3' ends of the *HSP150* gene, using LRE45 and LRE46 or LRE47 and LRE48 respectively, from the DNA from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc.).

Conditions were as follows: 1 µg/ml genomic DNA, $\approx 1.2 \times 10^{-10}$ moles of each primer, denature at 94°C for 61 seconds, anneal at 37°C for 121 seconds, DNA synthesis at 72°C for 181 seconds for 30 cycles, with a 10 second extension to the DNA synthesis step after each cycle, followed by a 4°C soak. PCR was carried out using a Perkin-Elmer-Cetus Thermal cycler and a Perkin-Elmer-Cetus PCR kit was used according to the manufacturer's recommendations. PCR products were analysed by gel electrophoresis and were found to be of the expected size. Each PCR product was digested with *EcoRI* and *PstI* and cloned into *EcoRI/PstI* digested pUC19 (Yanisch-Perron *et al*, 1985) to form pAYE503 (containing the 5' end of the *HSP150* gene) and pAYE504 (containing the 3' end of the *HSP150* gene) (see Fig. 1).

Plasmid DNA sequencing was carried out on pAYE503 and pAYE504 to

confirm that the inserts were the desired sequences. pAYE503 and pAYE504 were digested with *EcoRI* and *HindIII* and the *HSP150* gene fragments were isolated and cloned together into pUC19XH (a derivative of pUC19 lacking a *HindIII* site in its polylinker) to form pAYE505. The *URA3* gene was isolated
5 from YEp24 (Botstein *et al.*, 1979) as a *HindIII* fragment and cloned into the *HindIII* site of pAYE505 to form pAYE506 (Fig. 1). pAYE506 contains a selectable marker (*URA3*) flanked by 5' and 3' regions of the *HSP150* gene.

To construct a strain lacking the capacity to produce HSP150, a *ura3* derivative
10 of DB1 cir^o pAYE316 (Sleep *et al.*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al.*, 1987). Plasmid pAYE316 is based on the 2 μ m plasmid and contains a coding sequence for human albumin under the control of the yeast *PRB1* promoter, with an *ADHI* terminator and a *LEU2* selectable marker.

15

The strain was grown overnight in 100mL buffered minimal medium (Yeast Nitrogen Base [without amino acids, without ammonium sulphate, Difco], $(\text{NH}_4)_2\text{SO}_4$ 5g/L, citric acid monohydrate 6.09g/L, NaHPO_4 20.16g/L, sucrose 20g/L, pH6.5) and the cells were collected by centrifugation and then washed
20 once with sterile water. The cells were then resuspended in 10mL sterile water and 2mL aliquots were placed in separate 15mL Falcon tubes. A 5mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0 μ L, 20 μ L, 40 μ L, 80 μ L or 160 μ L. The cells were then incubated at 30°C for 20 min and then centrifuged and washed three times with
25 sterile water. Finally, the cells were resuspended in 1mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were
30 grown on YEP plates containing 2% w/v sucrose and then replica-plated onto

YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1mg/mL) and uracil (50µg/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by
5 introduction of the *URA3* gene by transformation.

The *ura3* strain, DBU3 cir^o (pAYE316), was transformed with *EcoRI* digested pAYE506 and Ura⁺ transformants were selected. The disruption of the *HSP150* gene in these transformants was confirmed by Southern blot analysis
10 using a fragment comprising the 5' and 3' ends of the *HSP150* gene (the *EcoRI* fragment from pAYE505) as a probe.

The yeast was then grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working
15 volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500
20 g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 ± 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

Table 1. Salts Mixture

5

Chemical	Concentration (g/L)
KH_2PO_4	114.0
MgSO_4	12.0
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	3.0
Na_2EDTA	2.0

Table 2. Trace Elements Solution

10

15

20

Chemical	Concentration (g/L)
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	3.0
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10.0
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	3.2
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.079
H_3BO_3	1.5
KI	0.2
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.5
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.56
H_3PO_4	75mL/L

Table 3. Vitamins Solution

25

30

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
<i>m</i> -inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, $(\text{NH}_4)_2\text{SO}_4$ 5 g/L, citric acid monohydrate 6.09 g/L, Na_2HPO_4 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun, Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h^{-1}). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved.

15

The fermentation broth was centrifuged to remove the cells and then subjected to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0. The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

25

Analysis of rHA purified from *Hsp150* strains revealed that no HSP150 protein was present in these samples. HSP150 protein is determined using prior art techniques such as ELISA or Western blotting.

30 Anti-HSP150 antibodies are disclosed in Russo *et al* (1992) *Proc. Nat. Acad.*

Sci. (USA) 89, 3671-3675.

Example 2

- 5 The *HSP150* protein coding sequence was deleted by using alternative fragments of the cloned *HSP150* sequences as follows.

The *URA3 HindIII* fragment from YEp24 (see Example 1) was cloned into pIC19R (Marsh J.L. *et al* (1984) *Gene* 32, 481-485) at *HindIII* to form
10 pAYE601 and then excised as a *Sall/ClaI* fragment and inserted into pAYE505 at the *XhoI* and *ClaI* sites to form pAYE602 (Fig 2). This plasmid was digested with *EcoRI* and then used to transform DBU3 *cir*^o (pAYE316), selecting for Ura⁺ transformants. The disruption of the *HSP150* gene in these transformants was confirmed by Southern blot analysis as described in Example
15 1.

Thus, in this example, the whole of the *HSP150* coding sequence is removed, whereas in Example 1 the sequence is disrupted to yield non-functional protein.

20 Example 3

Southern blotting has revealed an Hsp150 homologue in *Hansenula polymorpha* (now called *Pichia angusta*). The *P. angusta* gene may be functionally deleted by ways analogous to those in Examples 1 and 2.

References

- Boeke, J. D. *et al* (1987) *Methods Enzymol.* **154**, 164-175.
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- 25 Sambrook, J. *et al* (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
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Wang, H.Y. *et al* (1979) *Biotechnology & Bioeng.* **21**, 975

Yanisch-Perron, C. *et al* (1985) *Gene* **33**, 103-119.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Delta Biotechnology Limited
- (B) STREET: Castle Court, Castle Boulevard
- (C) CITY: Nottingham
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast Strains

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9411356.0
- (B) FILING DATE: 07-JUN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..40
- (D) OTHER INFORMATION: /note = "Oligonucleotide for PCR amplification of 5' end of Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTATTTCTTA TTTCGGGAAT TCTTAAAGAC AAAAAAGCTC
40

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of the 5' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGCTGTGGTG CTGCAGATGA TGCGCTGG
28

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..30

(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of 3' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTACTTCCG CTTCTGCAGC CGCTACCTCC

30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..38

(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of the 3' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCGTGTAGC GAGGGAATTC TGTGGTCACG ATCACTCG

38

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2048 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 397..1638

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGTGATCTTA CTATTTCTTA TTTCGGAAAT TATTAAAGAC
AAAAAAGCTC ATTAATGGCT 60

TTCCGTCTGT AGTGATAAGT CGCCAACTCA GCCTAATTTT
TCATTTCTTT ACCAGATCAG 120

GAAAACTAAT AGTACAAATG AGTGTTTTCT CAAGCGGAAC
ACCACATTTT GAGCTAAATT 180

TAGATTTTGG TCAAAATAAG AAAGATCCTA AAAAAGGAAT
GGTTGGTGAA AAATTTATTA 240

GCTTGAATGG TAGGAATCCT CGAGATATAA AAGGAACACT
TGAAGTCTAA CGACAATCAA 300

TTTCGATTAT GTCCTTCCTT TTACCTCAAA GCTCAAAAAA
ATATCAATAA GAAACTCATA 360

TTCCTTTTCT AACCTAGTA CAATAATAAT AATATA ATG CAA
TAC AAA AAG ACT 414

Met Gln Tyr Lys Lys Thr
1 5

TTG GTT GCC TCT GCT TTG GCC GCT ACT ACA TTG GCC GCC
TAT GCT CCA 462

Leu Val Ala Ser Ala Leu Ala Ala Thr Thr Leu Ala Ala Tyr Ala Pro
10 15 20

TCT GAG CCT TGG TCC ACT TTG ACT CCA ACA GCC ACT TAC
AGC GGT GGT 510

25

Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr Ala Thr Tyr Ser Gly Gly
 25 30 35

GTT ACC GAC TAC GCT TCC ACC TTC GGT ATT GCC GTT CAA
 CCA ATC TCC 558
 Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile Ala Val Gln Pro Ile Ser
 40 45 50

ACT ACA TCC AGC GCA TCA TCT GCA GCC ACC ACA GCC TCA
 TCT AAG GCC 606
 Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr Thr Ala Ser Ser Lys Ala
 55 60 65 70

AAG AGA GCT GCT TCC CAA ATT GGT GAT GGT CAA GTC CAA
 GCT GCT ACC 654
 Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Ala Thr
 75 80 85

ACT ACT GCT TCT GTC TCT ACC AAG AGT ACC GCT GCC GCC
 GTT TCT CAG 702
 Thr Thr Ala Ser Val Ser Thr Lys Ser Thr Ala Ala Ala Val Ser Gln
 90 95 100

ATC GGT GAT GGT CAA ATC CAA GCT ACT ACT AAG ACT ACC
 GCT GCT GCT 750
 Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala
 105 110 115

GTC TCT CAA ATT GGT GAT GGT CAA ATT CAA GCT ACC ACC
 AAG ACT ACC 798
 Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr
 120 125 130

TCT GCT AAG ACT ACC GCC GCT GCC GTT TCT CAA ATC AGT
 GAT GGT CAA 846
 Ser Ala Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Ser Asp Gly Gln
 135 140 145 150

ATC CAA GCT ACC ACC ACT ACT TTA GCC CCA AAG AGC ACC
 GCT GCT GCC 894
 Ile Gln Ala Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala
 155 160 165

GTT TCT CAA ATC GGT GAT GGT CAA GTT CAA GCT ACC ACC

ACT ACT TTA 942
 Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Thr Leu
 170 175 180

GCC CCA AAG AGC ACC GCT GCT GCC GTT TCT CAA ATC GGT
 GAT GGT CAA 990
 Ala Pro Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln
 185 190 195

GTT CAA GCT ACT ACT AAG ACT ACC GCT GCT GCT GTC TTT
 CAA ATT GGT 1038
 Val Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Phe Gln Ile Gly
 200 205 210

GAT GGT CAA GTT CTT GCT ACC ACC AAG ACT ACT CGT GCC
 GCC GTT TCT 1086
 Asp Gly Gln Val Leu Ala Thr Thr Lys Thr Thr Arg Ala Ala Val Ser
 215 220 225 230

CAA ATC GGT GAT GGT CAA GTT CAA GCT ACT ACC AAG ACT
 ACC GCT GCT 1134
 Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
 235 240 245

GCT GTC TCT CAA ATC GGT GAT GGT CAA GTT CAA GCA ACT
 ACC AAA ACC 1182
 Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr
 250 255 260

ACT GCC GCA GCT GTT TCC CAA ATT ACT GAC GGT CAA GTT
 CAA GCC ACT 1230
 Thr Ala Ala Ala Val Ser Gln Ile Thr Asp Gly Gln Val Gln Ala Thr
 265 270 275

ACA AAA ACC ACT CAA GCA GCC AGC CAA GTA AGC GAT GGC
 CAA GTC CAA 1278
 Thr Lys Thr Thr Gln Ala Ala Ser Gln Val Ser Asp Gly Gln Val Gln
 280 285 290

GCT ACT ACT GCT ACT TCC GCT TCT GCA GCC GCT ACC TCC
 ACT GAC CCA 1326
 Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala Thr Ser Thr Asp Pro
 295 300 305 310

GTC GAT GCT GTC TCC TGT AAG ACT TCT GGT ACC TTA GAA
ATG AAC TTA 1374

Val Asp Ala Val Ser Cys Lys Thr Ser Gly Thr Leu Glu Met Asn Leu
315 320 325

AAG GGC GGT ATC TTA ACT GAC GGT AAG GGT AGA ATT GGT
TCT ATT GTT 1422

Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly Arg Ile Gly Ser Ile Val
330 335 340

GCT AAC AGA CAA TTC CAA TTT GAC GGT CCA CCA CCA CAA
GCT GGT GCC 1470

Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro Pro Pro Gln Ala Gly Ala
345 350 355

ATC TAC GCT GCT GGT TGG TCT ATA ACT CCA GAC GGT AAC
TTG GCT ATT 1518

Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro Asp Gly Asn Leu Ala Ile
360 365 370

GGT GAC AAT GAT GTC TTC TAC CAA TGT TTG TCC GGT ACT
TTC TAC AAC 1566

Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu Ser Gly Thr Phe Tyr Asn
375 380 385 390

TTG TAC GAC GAA CAC ATT GGT AGT CAA TGT ACT CCA GTC
CAC TTG GAA 1614

Leu Tyr Asp Glu His Ile Gly Ser Gln Cys Thr Pro Val His Leu Glu
395 400 405

GCT ATC GAT TTG ATA GAC TGT TAAGCAGAAA ACTATTAGTT
CTTTTATCCT 1665

Ala Ile Asp Leu Ile Asp Cys
410

GATGACTTTT TCTCATTTGC ATTGATTAGA AAGGAAAAAA
AGAAGTGTCC TTTTCTACTA 1725

CTACTCTAGT CGCATCCATT CCTTTGCATT TATCTTTTCT
GCGGTTGGCC AATCCATTCT 1785

TCCGAGAATT TGGCTAGCCA TACTTGATGT TTTCCCATTA
TTGGTTCGTT TGGCAATGCT 1845

28

AATTTTCTTA ATTGCCCCTT ATATACTCTT CCATAAAATG
TTTTTTTTAT AACTAATTTT 1905

CTGTATATCA TTATCTAATA ATCTTATAAA ATGTTAAAAA
GACTTGGAAG GCAACGAGTG 1965

ATCGTGACCA CATAATTGCC TCGCTACACG GCAAAAATAA
GCCAGTCCTA ATGTGTATAT 2025

TAAAGGCTGC ATGTGGCTAC GTC
2048

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 413 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Gln Tyr Lys Lys Thr Leu Val Ala Ser Ala Leu Ala Ala Thr Thr
1 5 10 15

Leu Ala Ala Tyr Ala Pro Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr
20 25 30

Ala Thr Tyr Ser Gly Gly Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile
35 40 45

Ala Val Gln Pro Ile Ser Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr
50 55 60

Thr Ala Ser Ser Lys Ala Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly
65 70 75 80

Gln Val Gln Ala Ala Thr Thr Thr Ala Ser Val Ser Thr Lys Ser Thr
85 90 95

Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr
100 105 110

Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln
 115 120 125

Ala Thr Thr Lys Thr Thr Ser Ala Lys Thr Thr Ala Ala Ala Val Ser
 130 135 140

Gln Ile Ser Asp Gly Gln Ile Gln Ala Thr Thr Thr Thr Leu Ala Pro
 145 150 155 160

Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln
 165 170 175

Ala Thr Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala Val Ser
 180 185 190

Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
 195 200 205

Ala Val Phe Gln Ile Gly Asp Gly Gln Val Leu Ala Thr Thr Lys Thr
 210 215 220

Thr Arg Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr
 225 230 235 240

Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val
 245 250 255

Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Thr Asp
 260 265 270

Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Gln Ala Ala Ser Gln Val
 275 280 285

Ser Asp Gly Gln Val Gln Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala
 290 295 300

Ala Thr Ser Thr Asp Pro Val Asp Ala Val Ser Cys Lys Thr Ser Gly
 305 310 315 320

Thr Leu Glu Met Asn Leu Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly
 325 330 335

Arg Ile Gly Ser Ile Val Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro
 340 345 350

30

Pro Pro Gln Ala Gly Ala Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro
355 360 365

Asp Gly Asn Leu Ala Ile Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu
370 375 380

Ser Gly Thr Phe Tyr Asn Leu Tyr Asp Glu His Ile Gly Ser Gln Cys
385 390 395 400

Thr Pro Val His Leu Glu Ala Ile Asp Leu Ile Asp Cys
405 410

CLAIMS

1. A process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the desired protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).
5
2. A process according to Claim 1 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.
- 10 3. A process according to Claim 2 wherein substantially no Hsp150 protein is produced.
4. A process according to any one of the preceding claims wherein the desired protein is an albumin.
15
5. A process according to Claim 4 wherein the desired protein is a human albumin.
6. A process according to any one of the preceding claims wherein the yeast is a *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces*, *Pichia* or
20 *Saccharomyces* species.
7. A process according to Claim 6 wherein the yeast is *S. cerevisiae*.
- 25 8. A process according to any one of the preceding claims wherein the desired protein is secreted from the yeast into the surrounding medium and purified therefrom.
9. A protein prepared by a process according to any one of the preceding
30 claims.

10. A culture medium containing a desired protein and prepared by a process according to any one of Claims 1 to 7.
- 5 11. A yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in Hsp150.
12. A yeast according to Claim 11 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.
- 10 13. A yeast according to Claim 11 wherein substantially no Hsp150 protein is produced by the yeast.
14. A yeast according to any one of Claims 11 to 13 wherein the desired protein is an albumin.
- 15 15. A yeast according to Claim 14 wherein the desired protein is a human albumin.
- 20 16. A yeast according to any one of Claims 11 to 15 wherein the yeast is *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces* or *Saccharomyces* species.
17. A yeast according to Claim 16 wherein the yeast is *S. cerevisiae*.
- 25 18. A yeast according to any one of Claims 11 to 17 wherein the yeast is transformed with a DNA construct such that the desired protein is secreted from the yeast during culturing thereof.
- 30 19. A method of preparing a yeast according to any one of Claims 11 to 18

comprising the steps of

(i) transforming the yeast with a coding sequence for expression of the desired protein, and

5

(ii) disrupting the genome of the yeast such that the yeast has an abnormally low level of Hsp150,

10

wherein steps (i) and (ii) may be carried out in either order or simultaneously.

5' and 3' regions of *HSP150* gene obtained by PCR:

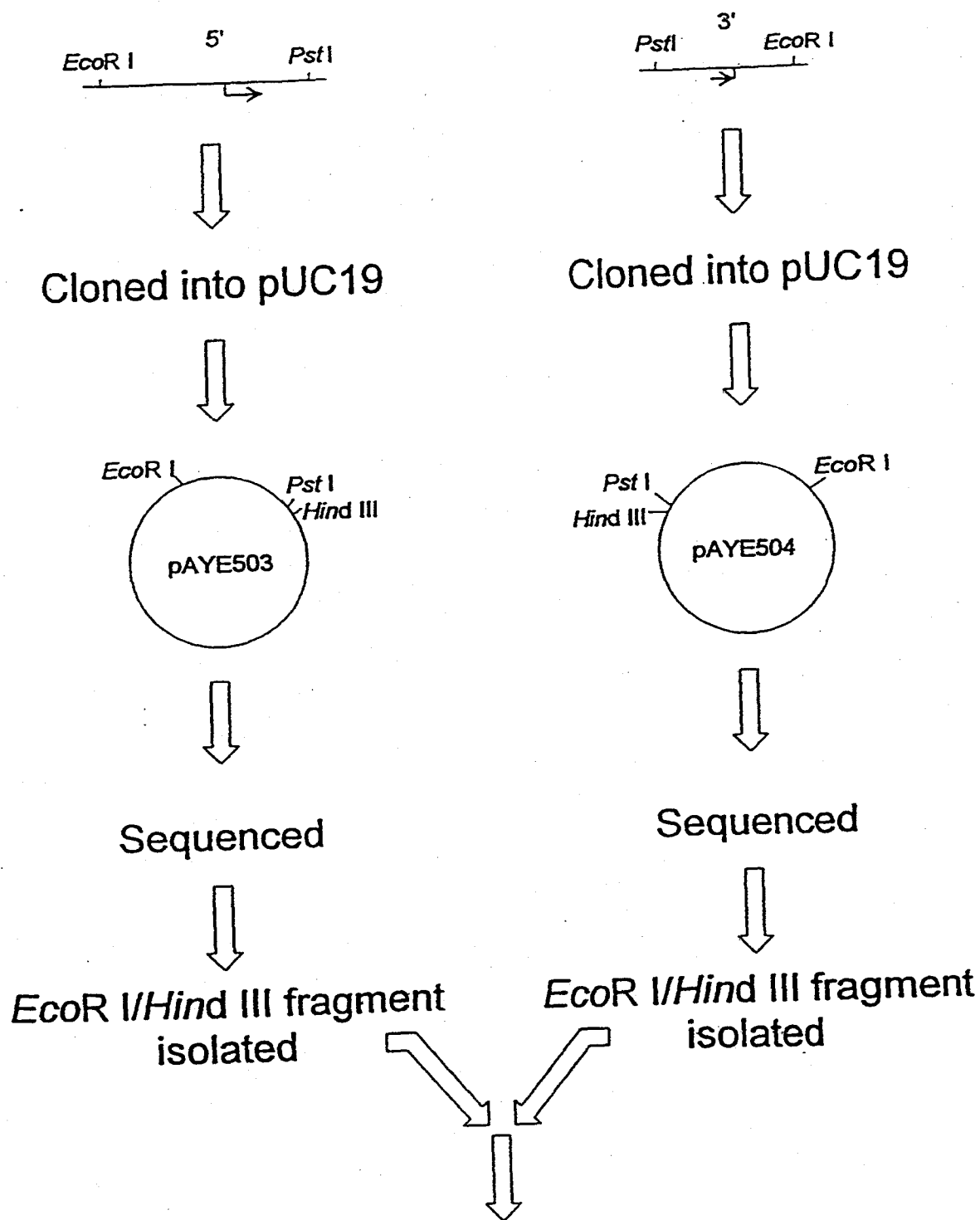


Figure 1

Figure 1 cont'd

Subcloned into pUC19HX

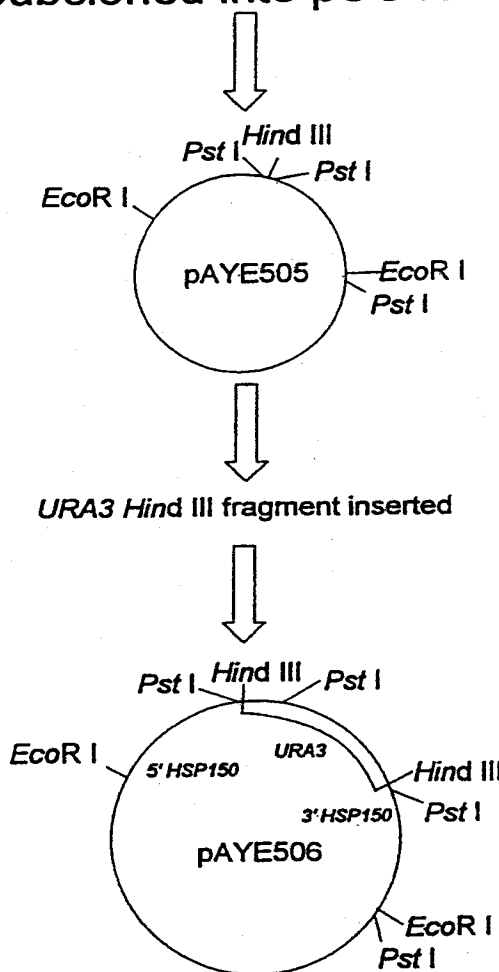
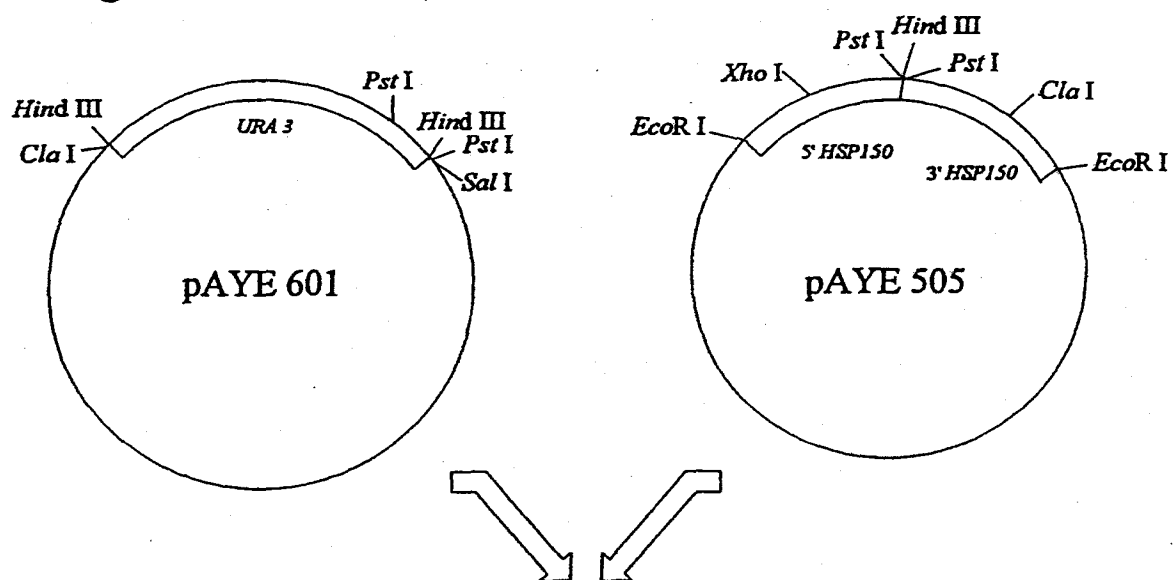
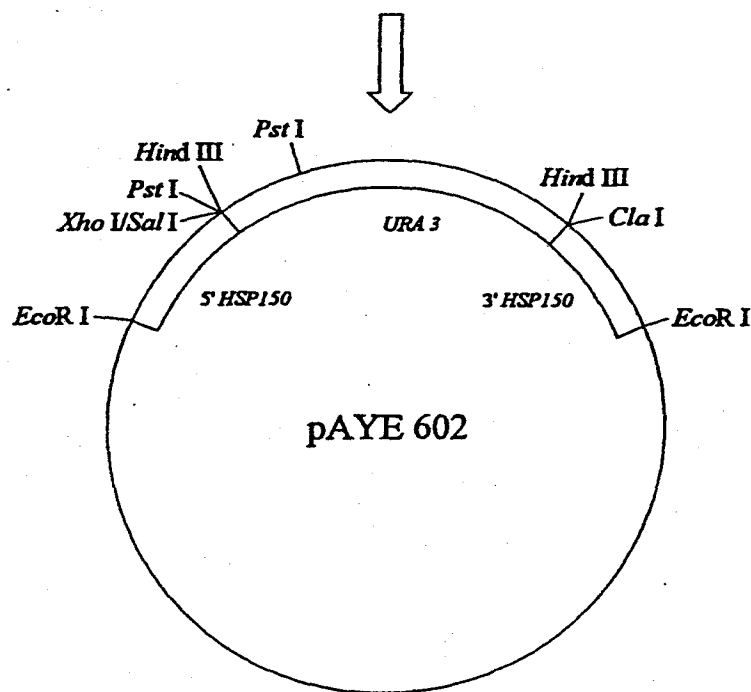


Fig 2



Ligate *Cla* I/ *Sal* I *URA* 3 fragment into *Cla* I/*Xho* I cut pAYE 505



INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/GB 95/01317

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/14 C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOL GEN GENET, MAY 1993, 239 (1-2) P273-80, GERMANY, RUSSO P ET AL 'Dual regulation by heat and nutrient stress of the yeast HSP150 gene encoding a secretory glycoprotein.' see the whole document ---	1,2, 6-12, 16-19
X	BIOTECHNOLOGY, vol. 8, 1990 NEW YORK US, pages 42-46, SLEEP D. ET AL. 'The secretion of human serum albumin from the yeast Sachcharomyces cerevisiae using five different leader sequences' cited in the application see the whole document -----	9,10

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

28 September 1995

Date of mailing of the international search report

10.10.95

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(12) 公開特許公報 (A)

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C 0 7 K 14/40		C 0 7 K 14/40	4 B 0 5 0
		19/00	4 B 0 6 4
C 1 2 N 1/19		C 1 2 N 1/19	4 B 0 6 5
9/14		9/14	4 H 0 4 5

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(21) 出願番号 特願平10-251526

(22) 出願日 平成10年9月4日 (1998.9.4)

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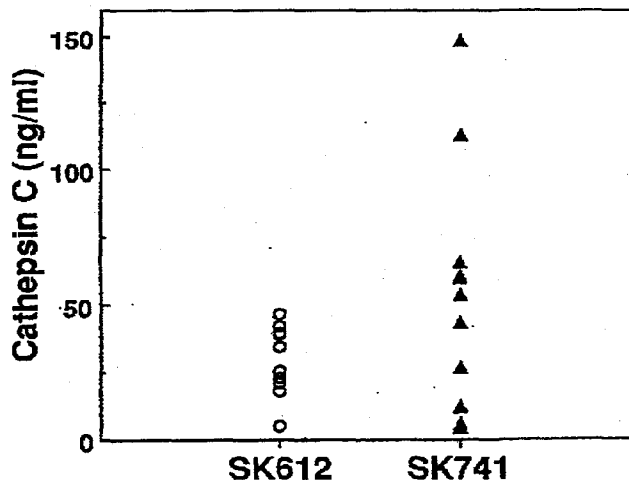
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(54) 【発明の名称】 プロテアーゼ活性の低下したカンジダ・ボイジニ株及び異種タンパク質製造用宿主としてのその使用

(57) 【要約】

【課題】 異種遺伝子の高発現を可能にするカンジダ・ボイジニ (*Candida boidinii*) 株、それを利用する異種タンパク質の製造方法を提供すること。

【解決手段】 プロテアーゼ活性の低下したカンジダ・ボイジニ株、特にプロテイナーゼA、プロテイナーゼB又はその両方のプロテアーゼ活性が喪失されたカンジダ・ボイジニ株、このカンジダ・ボイジニ株を、有用な異種タンパク質 (例えばカテプシンC) をコードする遺伝子を含む発現ベクターで形質転換して異種タンパク質を製造する方法、プロテイナーゼA活性又はプロテイナーゼB活性を有するタンパク質、それらのタンパク質をコードするDNA、並びにカンジダ・ボイジニ由来プロテイナーゼAの分泌シグナルペプチド。



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【特許請求の範囲】

【請求項1】 プロテアーゼ活性の低下したカンジダ・ボイジニ (*Candidaboidinii*) 株。

【請求項2】 プロテイナーゼA、プロテイナーゼB又はその両方のプロテアーゼ活性が喪失された、請求項1に記載のカンジダ・ボイジニ株。

【請求項3】 カンジダ・ボイジニSK740株、SK741株、SK774株又はSK775株である、請求項2に記載のカンジダ・ボイジニ株。

【請求項4】 請求項1～3のいずれかに記載のカンジダ・ボイジニ株を、有用な異種タンパク質をコードする遺伝子を含む発現ベクターで形質転換し、適当な培地にて培養し、生成した異種タンパク質を回収することを含む、タンパク質の製造方法。

【請求項5】 異種タンパク質がカテプシンCである、請求項4に記載の方法。

【請求項6】 発現ベクターが、異種タンパク質をコードする遺伝子の5'末端に隣接して分泌シグナルペプチド配列をコードするDNAを含む、請求項4又は5に記載の方法。

【請求項7】 分泌シグナルペプチド配列がプロテアーゼタンパク質由来のものである、請求項6に記載の方法。

【請求項8】 分泌シグナルペプチド配列が配列番号4に示すアミノ酸配列からなる、請求項7に記載の方法。

【請求項9】 配列番号2に示される23位～420位のアミノ酸配列、あるいはその配列において少なくとも80%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有し、かつプロテアーゼ活性を有するカンジダ・ボイジニ株由来のプロテイナーゼA又はその誘導体。

【請求項10】 請求項9に記載のカンジダ・ボイジニ株由来のプロテイナーゼA又はその誘導体をコードするDNA。

【請求項11】 配列番号2に示されるアミノ酸配列、あるいはその配列において少なくとも80%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有する、カンジダ・ボイジニ株由来の前駆体プロテイナーゼA又はその誘導体。

【請求項12】 請求項11に記載のカンジダ・ボイジニ株由来の前駆体プロテイナーゼA又はその誘導体をコードするDNA。

【請求項13】 配列番号3に示される塩基配列を有する、請求項12に記載のDNA。

【請求項14】 配列番号5に示されるアミノ酸配列、あるいはその配列において少なくとも80%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有し、かつプロテアーゼ活性を有するカンジダ・ボイジニ株由来のプロテ

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ナーゼB又はその誘導体。

【請求項15】 請求項14に記載のカンジダ・ボイジニ株由来のプロテイナーゼB又はその誘導体をコードするDNA。

【請求項16】 配列番号6に示される塩基配列を有する、請求項15に記載のDNA。

【請求項17】 配列番号4に示されるアミノ酸配列からなる、カンジダ・ボイジニ由来プロテイナーゼAの分泌シグナルペプチド。

【請求項18】 カンジダ・ボイジニSK741株。

【請求項19】 カンジダ・ボイジニSK741株を、カテプシンCをコードする遺伝子を含む発現ベクターで形質転換し、適当な培地にて培養し、生成したカテプシンCを回収することを含む、カテプシンCの製造方法。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】 本発明は、カンジダ・ボイジニ (*Candida boidinii*) のプロテアーゼ遺伝子、該プロテアーゼ遺伝子を改変したDNAを有するカンジダ・ボイジニ株、該カンジダ・ボイジニ株を宿主として用いる異種タンパク質の製造法に関する。このカンジダ・ボイジニ株を宿主とするタンパク質発現系を用いれば、目的異種タンパク質を収率よく生産することができる。また、本発明は、カンジダ・ボイジニを宿主とする異種タンパク質の分泌発現に有用なシグナルペプチドに関し、該シグナルペプチドを利用する異種タンパク質の分泌発現系、及び該分泌発現系を用いる異種タンパク質の製造法に関する。

【0002】

【従来の技術】 メタノール資化性酵母カンジダ・ボイジニは、近年、異種タンパク質発現系の有効な宿主として開発されてきた。メタノール資化経路に存在するアルコールオキシダーゼ、ジヒドロキシアセトンシンターゼ、ギ酸脱水素酵素はメタノール存在下で培養すると、著量生産され、それらの遺伝子の調節領域を用いた異種遺伝子の発現方法が研究されている（特開平5-344895号公報、国際公開第WO 97/10345号等）。しかしながら異種タンパク質を遺伝子組み換え法によって生産する場合、目的産物が宿主由来のプロテアーゼによって分解されることがある。そのような場合、目的タンパク質の生産量が減少し、またタンパク質分解産物の混入により目的タンパク質の精製が困難となる。

【0003】 遺伝子組み換え法によって生産される目的タンパク質の分解の問題を回避するために、目的タンパク質を分解するプロテアーゼ活性を阻害するような培養方法が用いられてきた。例えば組換え体を培養する培地のpHを調整することによりプロテアーゼ作用を阻害することが可能である。しかしながらこの方法はある種の異種タンパク質を発現する宿主酵母の増殖に影響を与えるであろうし、細胞外でのタンパク質の分解にのみ効果

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的である。

【0004】一方、酵母 *Saccharomyces cerevisiae*、*Pichia pastoris* においてプロテイナーゼA、プロテイナーゼBを不活性化した株をプロテアーゼ欠損株として用いることにより、菌体内及び菌体外タンパク質生産を増加させたという例が示されている（特表平6-506117号公報、Weis, H. M. ら, *FEBS Lett.*, 377, 451 (1995)、Inoue, K. ら, *Plant Cell Physiol.*, 38 (3), 366 (1997)）。

【0005】プロテイナーゼA及びプロテイナーゼBは液胞に局在するプロテアーゼで、それぞれPEP4遺伝子、PRB1遺伝子によってコードされている。酵母 *Saccharomyces cerevisiae* の研究によれば、プロテイナーゼA及びプロテイナーゼBは自分自身やカルボキシペプチダーゼYなどの別のプロテアーゼを活性化する（vandenHazel, H. B. ら, *YEAST*, 12, 1 (1996)）。ところで、カンジダ・ボイジニを用いて異種遺伝子を発現させる際、タンパク質生産量を高めるためにプロテアーゼ欠損株を用いることについては全く知られていなかったが、また当業者においてそのような想起を拒む以下に示すような問題点があった。

【0006】*Saccharomyces cerevisiae* や *Pichia pastoris* とカンジダ・ボイジニとは菌学的に本質的に異なっていて、多くの代謝的、生理的相違が存在する。それゆえタンパク質分解機構が異なっていることは容易に推測される。今のところカンジダ・ボイジニに存在するタンパク質分解活性についての知見は全く存在せず、本発明者らによって今回初めて、*Saccharomyces cerevisiae*、*Pichia pastoris* ではプロテイナーゼA遺伝子欠損によって完全に活性を失うカルボキシペプチダーゼYが、プロテイナーゼA遺伝子を欠損したカンジダ・ボイジニにおいては約40%活性が残存していることが見い出されたが、この例もカンジダ・ボイジニのタンパク質分解機構が前記の他の2つの酵母のものとは異なることを示すものである。

【0007】プロテアーゼ欠損株の取得は、変異株のスクリーニング、遺伝子破壊によって取得される。変異株をスクリーニングするためには膨大な数の変異株について解析しなければならず、*Saccharomyces cerevisiae*、*Pichia pastoris* と異なり、胞子形成能のないカンジダ・ボイジニではかけ合せにより目的とする遺伝子だけに変異が導入されたかどうか解析することは不可能である。さらに変異した形質が変異前の状態に戻る復帰変異株も起こり得る。一方、遺伝子破壊法は目的とする遺伝子だけを欠損させることが可能であるため、有効な手法であるが、遺

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伝子破壊を行うために宿主の目的遺伝子領域を獲得しなければならない。しかしながら、カンジダ・ボイジニに関するこのようなプロテアーゼ及びその遺伝子に関する知見は現在まで全く得られていない。したがって、カンジダ・ボイジニのプロテアーゼ欠損株を利用した発現系における生産性の向上について、これまで何ら検討されてこなかった。

【0008】

【発明が解決しようとする課題】本発明は、カンジダ・ボイジニに由来するプロテアーゼ、そのプロテアーゼをコードする塩基配列を有するDNA、及びそのプロテアーゼ遺伝子の欠失したカンジダ・ボイジニ株を提供することを目的とする。またそのプロテアーゼタンパク質に由来する分泌シグナルペプチドをコードするDNA配列を酵母分泌発現系に有用なシグナルペプチド、及びそのシグナルペプチドを利用した異種タンパク質の分泌生産方法を提供することを目的とする。

【0009】

【課題を解決するための手段】本発明者らは、メタノール資化性酵母 *Candida boidinii* のプロテアーゼ遺伝子を解析し、異種遺伝子の効率的発現を達成すべく鋭意研究を行った結果、プロテアーゼ遺伝子欠失カンジダ・ボイジニ株を用いて異種遺伝子の高発現を達成することにより、本発明を完成するに至った。本発明は、プロテアーゼ活性の低下したカンジダ・ボイジニ株を提供する。ここで、プロテアーゼは組換え技術での発現によって生成された異種タンパク質の分解に関与するものであるのが好ましい。その種のプロテアーゼの少なくとも1つの活性を喪失させることによって、全体的にプロテアーゼ活性を低下させることができる。

【0010】本発明の実施態様において、本発明はプロテイナーゼA、プロテイナーゼB又はその両方のプロテアーゼ活性が喪失されたカンジダ・ボイジニ株を提供する。より具体的には、そのような菌株はカンジダ・ボイジニSK740株、SK741株、SK774株又はSK775株である。本発明はまた、上記の本カンジダ・ボイジニ株を、有用な異種タンパク質をコードする遺伝子を含む発現ベクターで形質転換し、適当な培地にて培養し、生成した異種タンパク質を回収することを含む、タンパク質の製造方法を提供する。

【0011】本発明の実施態様において、本発明には、カンジダ・ボイジニSK741株を、カテブシンCをコードする遺伝子を含む発現ベクターで形質転換し、適当な培地にて培養し、生成したカテブシンCを回収することを含む、カテブシンCの製造方法が包含される。発現ベクターは、異種タンパク質をコードする遺伝子の5'末端に隣接して分泌シグナルペプチド配列をコードするDNAを含むことができる。シグナルペプチドはゴルジ体で合成された前駆体タンパク質を膜に輸送する役割を有し、シグナルペプチダーゼにより切断され、結果的に

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成熟タンパク質が細胞外に分泌されることになる。本発明の実施態様において、分泌シグナルペプチド配列はプロテアーゼタンパク質由来のもの、より具体的にはカンジダ・ボイジニのプロテイナーゼA由来の配列番号4に示すアミノ酸配列からなる。

【0012】本発明はさらに、配列番号2に示される23位～420位のアミノ酸配列、あるいはその配列において少なくとも80%、好ましくは少なくとも90%、より好ましくは少なくとも95%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有し、かつプロテアーゼ活性を有するカンジダ・ボイジニ株由来のプロテイナーゼA又はその誘導体を提供する。本発明はさらにまた、上記のカンジダ・ボイジニ株由来のプロテイナーゼA又はその誘導体をコードするDNAをも提供する。

【0013】本発明はまた、配列番号2に示されるアミノ酸配列、あるいはその配列において少なくとも80%、好ましくは少なくとも90%、より好ましくは少なくとも95%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有する、カンジダ・ボイジニ株由来の前駆体プロテイナーゼA又はその誘導体を提供する。本発明はさらに、上記のカンジダ・ボイジニ株由来の前駆体プロテイナーゼA又はその誘導体をコードするDNAをも提供する。具体的には、該DNAは配列番号3に示される塩基配列を有することができる。

【0014】本発明はまた、配列番号5に示されるアミノ酸配列、あるいはその配列において少なくとも80%、好ましくは少なくとも90%、より好ましくは少なくとも95%の相同性を有するように1個以上のアミノ酸が欠失、置換、挿入及び／又は付加されたアミノ酸配列を有し、かつプロテアーゼ活性を有するカンジダ・ボイジニ株由来のプロテイナーゼB又はその誘導体を提供する。本発明はさらに、上記のカンジダ・ボイジニ株由来のプロテイナーゼB又はその誘導体をコードするDNAをも提供する。具体的には、該DNAは配列番号6に示される塩基配列を有する。

【0015】カンジダ・ボイジニ株のプロテイナーゼA、B又は前駆体プロテイナーゼA、B及びそれらをコードするDNAは、パン酵母 (*Saccharomyces cerevisiae*) 又はピキア・パストリス (*Pichia pastoris*) のプロテイナーゼA、B及びそれらをコードするPEP4、PRB1遺伝子と配列上の類似性をもつが、そのアミノ酸及びDNA配列は*Saccharomyces cerevisiae*や*Pichia pastoris*のプロテイナーゼA及びPEP4、PRB1遺伝子とはともに80%より低い相同性を有し本質的に異なるものである。それゆえ、本発明における上記の「誘導体」は、目的とするプロテアーゼ活性が得られる限り、及び／又は、配列番号2、3又は配列番号5、6に示される配列に、それらの配列と少なくとも80%、

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好ましくは少なくとも90%、より好ましくは少なくとも95%の相同性を有する限り、置換、欠失、挿入又は付加等の変異を含み得ることを意味する。また、本発明の誘導体には、配列番号2又は5に示されるアミノ酸配列と本質的に同一のアミノ酸配列をコードする任意の塩基配列をもつDNAや染色体上の相同遺伝子を破壊するのに十分な相同性をもつDNA配列も含まれる。例えば、配列番号3で示される塩基配列の第6番目の「g」が「a」に置換されても、本発明の目的とするプロテアーゼ活性が得られる限り、かかる置換された配列も本発明に含まれることを意味する。この点で、本発明の誘導体は、タンパク質及びDNAの両方において、配列番号2、3、5又は6に示されるアミノ酸配列又は塩基配列を実質的に含む配列を有する、と表現することも可能である。本発明はまた、配列番号4に示されるアミノ酸配列からなる、カンジダ・ボイジニ由来プロテイナーゼAの分泌シグナルペプチドを提供する。

【0016】

【発明の実施の形態】本発明により、カンジダ・ボイジニのプロテアーゼをコードする塩基配列が該プロテアーゼの産生が少なくとも抑制されるように改変（置換、欠失、挿入、付加、等）されたDNA、好ましくは該プロテアーゼをコードする塩基配列に形質転換マーカー遺伝子が挿入されたDNA、並びに該改変DNAを有することによりタンパク質分解活性が親株に対して著しく低下したプロテアーゼ遺伝子欠失カンジダ・ボイジニ株が提供される。

【0017】このような株の例としては、野生型のプロテイナーゼAをコードするPEP4遺伝子が上述のように改変されたPEP4遺伝子で置換されたカンジダ・ボイジニ株であり、該株では野生型プロテイナーゼAを全く産生しないのみならず、本来、プロテイナーゼAにより活性化されるカルボキシペプチダーゼYやプロテイナーゼBなどのプロテアーゼ活性も著しく抑制されている。また、PEP4遺伝子に加えてプロテイナーゼBをコードするPRB1遺伝子が上述のように改変されたPRB1遺伝子で置換されたカンジダ・ボイジニ株も作出可能であり、このような二重変異株においては、PEP4遺伝子欠失株ではわずかに活性が残存するとされるプロテイナーゼB活性も全く検出されないことが期待される。プロテアーゼ活性の低下したカンジダ・ボイジニ株、PEP4遺伝子欠失株とPEP4、PRB1遺伝子欠失株は、栄養培地を用いた培養条件下で野生株と同等の増殖能力を保持しているという特徴を有する。このことはこれらの遺伝子の有無は栄養条件下ではカンジダ・ボイジニの増殖に影響を及ぼさないことを意味する。従って本発明によるプロテアーゼ活性の抑制された酵母は異種タンパク質生産のための優れた宿主である。特に当該酵母はプロテアーゼ感受性の異種タンパク質を効率的に生産することができる。

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【0018】本発明により、さらに、上記の本カンジダ・ボイジニ株を異種タンパク質をコードする遺伝子（即ち、異種遺伝子）を含む発現ベクターで形質転換して得られた形質転換体を培養し、菌体又は培養上清から目的タンパク質を回収することを含む、異種タンパク質の製造方法が提供される。ここで異種遺伝子とは、発現の対象となる任意の遺伝子を意味し、例えば、カテプシンC、表皮増殖因子（EGF）、インシュリン様増殖因子1（IGF-1）、ヒト血清アルブミン、エリスロポイエチン（EPO）、スロンボポイエチン（TPO）等が

10 挙げられるが、これらに限定されない。また、異種遺伝子はいかなる手法によって得られるものであってもよい。

【0019】また、発現ベクターは、異種タンパク質の分泌のためのシグナルペプチド配列をコードするDNAを、該異種タンパク質をコードする遺伝子の5'末端に隣接（即ち、フランキング）して含むことができる。これによって、発現によって生成した異種タンパク質を細胞外へ分泌させることを可能とする。この分泌シグナルペプチドとしては、例えば、カテプシンCが本来有するシグナル配列、パン酵母の α 因子の分泌シグナルペプチド配列、カンジダ・ボイジニのプロテイナーゼAのシグナルペプチドが利用できる。

【0020】本発明の実施態様において、異種遺伝子として、ウシ由来のジペプチジルプロテアーゼであるカテプシンCをコードする遺伝子が例示される。本酵素は、タンパク質のN末端からアミノ酸を2つずつ分解するプロテアーゼであり、産業上有用な酵素である。この新規遺伝子は配列番号8に示されたアミノ酸配列と実質的に同一のアミノ酸配列を有するタンパク質であると特徴付けられる。カテプシンCは不活性の前駆体（プレプロ体）として発現し、その後のプロセッシングにより分泌シグナルとして機能するプレペプチド領域が切断され、続いてプロペプチド領域が切断されて活性を有する成熟タンパク質になる。プロペプチド領域とは一般にタンパク質の不活性前駆体に含まれるペプチド断片であり、不活性前駆体から該ペプチドが特異的なプロテアーゼ等により切断除去されてそのタンパク質特有の活性を示す。従ってウシカテプシンCのプロペプチド配列とそれに続く成熟タンパク質を構成するポリペプチド配列が、該酵素の分泌過程での活性化にとって必要である。このために、該酵素の酵母における分泌発現には、分泌のためのシグナル配列をカテプシンCのプロペプチドのN末端に付加することが必要である。この分泌シグナルペプチドとしては上記例示のもの、好ましくはカンジダ・ボイジニのプロテイナーゼAのシグナルペプチドを利用できる。また該タンパク質の発現にとって、上記のプロテアーゼ遺伝子欠損株は特に有用であった。

【0021】従って、本発明には、カテプシンCを生産するカンジダ・ボイジニ株、好適にはプロテアーゼ活性

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の低下したカンジダ・ボイジニ株、並びに、プロテアーゼ活性の低下したカンジダ・ボイジニ株を用いたウシ由来カテプシンCの製造方法も包含される。以下、本発明をさらに詳細に説明する。

【0022】本発明者らは上記課題を解決するために、

（1）カンジダ・ボイジニのプロテイナーゼA及びプロテイナーゼB遺伝子の塩基配列を解明し、（2）プロテアーゼ遺伝子破壊プラスミドを構築し、（3）本プラスミドを用いて形質転換体を作製し、プロテアーゼ遺伝子欠失カンジダ・ボイジニ株を取得した。さらに（4）プロテアーゼ遺伝子欠失カンジダ・ボイジニ株を宿主として異種遺伝子を発現させた時、その生産量が野生株を宿主としたときよりも優れていることを確認し、本発明を完成するに至ったものである。

【0023】（1）プロテイナーゼA及びプロテイナーゼB遺伝子

本発明の遺伝子を取得するための出発材料としては、カンジダ・ボイジニATCC48180株が例示される。本発明においてクローニング工程は、公知の方法（Molecular Cloning (1989), Methods in Enzymology 194 (1991))に従って行なうことが出来る。すなわち、（a）上記酵母の全DNAに由来するDNA断片、もしくは上記酵母のmRNAより合成されたcDNA断片を組み込んだ遺伝子導入用ベクターを宿主に導入して上記酵母の遺伝子ライブラリーを作製する。（b）ついで、かかる遺伝子ライブラリーから所望のクローンを選択して、当該クローンを増幅することにより上記のクローニング工程を実施することが出来る。

【0024】（a）酵母の遺伝子ライブラリーの調製
酵母の全DNAの抽出は、例えば酵母のプロトプラストを調製して、当該プロトプラストから、通常公知のDNA抽出法、すなわち細胞残さを除去した後、高塩濃度下でDNAをアルコール沈殿し、さらにフェノールやクロロホルム抽出後にアルコール沈殿して精製する方法を用いて行なうことが出来る。なお、上記の予めプロトプラストを調製する方法の他に、ガラスビーズ等による細胞破砕法等によってもDNAの抽出を行なうことが出来るが、高分子量のDNAを調製することが容易であるという点から上記プロトプラスト法を行なうのが好ましい。

得られた染色体DNAを適当な制限酵素によって消化し、適当なベクターに連結した後、適当な大腸菌宿主に形質転換することによってゲノミックライブラリーを得ることができる。

【0025】この際用いられるベクターとしては、通常公知の遺伝子ライブラリー調製用ベクターとして知られる、pBR系統、pUC系統、ブルースクリプト（Blue script）系統等の一般に市販されている入手可能なプラスミドを用いることも出来る。また、gt系統やEMBL系統のファージベクターあるいはコスミド等も広く用いることが出来る。調製した遺伝子ライブラ

リー作製用ベクターで形質転換もしくは形質導入を行なう宿主は、上記ベクターの種類に応じたものを採用することが出来る。

【0026】(b) クローンの選択

上記遺伝子ライブラリーから、所望のプロテイナーゼA及びプロテイナーゼB遺伝子を有するクローンをそれぞれの遺伝子に特有の配列を含む標識プローブを用いてコロニー・ハイブリダイゼーション法、ブランク・ハイブリダイゼーション法等により選択し、取得することが出来る。

【0027】プローブに用いるプロテイナーゼA及びプロテイナーゼB遺伝子に特有の配列は、各種起源のプロテアーゼで保存されているアミノ酸配列より、カンジダ・ボイジニのコドン使用頻度を参考にプライマーを設計し、カンジダ・ボイジニの染色体DNAを鋳型とするPCR法により、所望するDNA断片を特異的に増幅して取得される。またカンジダ・ボイジニから精製した該プロテアーゼのアミノ酸配列に対応する2組のオリゴヌクレオチドを合成し、それらをプライマーとしてカンジダ・ボイジニの染色体DNAを鋳型とするPCR法により、所望するDNA断片を特異的に増幅して取得することも可能である。なお、合成したオリゴヌクレオチドをプローブとして用いることもできる。

【0028】上記方法により得られる所望の遺伝子の塩基配列の決定および確認は、例えばマクサム・ギルバートの化学変法(Maxam-Gilbert, Methods in Enzymology, 65, 499 (1980))やジデオキシヌクレオチド鎖終結法(Messing, J. と Vieire, J., Gene, 19, 269 (1982))及びその自動化された変法等により行ない得る。

【0029】(2) プロテアーゼ遺伝子破壊プラスミドの構築

プロテアーゼをコードするDNA配列を改変して、機能的プロテアーゼタンパク質が産生できないようにされたDNA、選択マーカー遺伝子等と共に適当なベクターの中に挿入され、遺伝子破壊プラスミドとして使用される。本プラスミドを用いた部位特異的組み込みにより、染色体上の該遺伝子が置換されることにより作製することができる。ここで用いる機能的プロテアーゼタンパク質が産生できないように改変されたDNA配列とは、タンパク質をコードするDNA配列の塩基が置換されたものか、一部分を欠失するか又は少なくとも1つのヌクレオチドが挿入(若しくは付加)されたものであり、これらの改変によってタンパク質をコードするDNA配列の読み枠がずれて発現されないか、発現されても得られる生成物の機能が変異することにより、本来のプロテアーゼ活性を有するタンパク質をコードできなくなる。好ましくはこのような改変されたDNA配列は、タンパク質をコードするDNA配列中に形質転換マーカー遺伝子などを挿入することによって作製することができる。このようなDNA断片を用いて染色体上の遺伝子を破壊する

ことができると共に導入された形質転換マーカー遺伝子を指標として改変されたプロテアーゼ遺伝子を有する変異体をスクリーニングできるという利点がある。ここで使用される選択マーカー遺伝子としては、G418等の抗生物質耐性遺伝子、URA3、LEU2等の宿主の栄養要求性を相補する遺伝子が例示される。発現ベクターの構成成分をベクターに挿入することは、後記実施例の記載を参照して、あるいは慣用の技術により当業者が容易に実施することが可能である。

10 【0030】(3) プロテアーゼ遺伝子欠失カンジダ・ボイジニ株の取得

プロテアーゼ活性が野生型株に比較して抑制されたカンジダ・ボイジニ株は、上述の機能的なプロテアーゼタンパク質が産生できないように改変されたDNA配列を用いて適切な酵母宿主を形質転換して内在性の遺伝子を置き換えることにより作製することができる。このための方法としては、遺伝子置換によって染色体上の標的遺伝子を物理的に除去して改変遺伝子と置換する方法がある。これは、標的遺伝子の5'側と3'側の領域と相同的な末端配列を有する直鎖状DNA断片、好ましくは、形質転換マーカー遺伝子などの挿入により分断された遺伝子を含む改変DNA断片を用いて酵母宿主を形質転換することにより達成される。この形質転換マーカー遺伝子として好ましくは自発的な組換えにより染色体から除去され得る改変したURA3遺伝子を用いることができる。この改変したURA3遺伝子とは、その5'側と3'側とに相同なDNA配列を同一方向に配置した構造としている。これにより、酵母染色体上に組み込まれた後にこの反復配列間での自発的な組換えが生じてURA3遺伝子が抜け落ちることが可能であり、形質転換遺伝子マーカーとしてURA3遺伝子を再利用することが可能となる。この際、Ura⁻株は5-フルオロオロチン酸(5-FOA)耐性となることから、5-FOA感受性のUra⁺株の中からURA3遺伝子が自発的な相同組換えにより抜け落ちたUra⁻株の選択は容易である。

【0031】また、この他の方法としてポップイン・ポップアウトとも呼ばれる方法(Rothstein R., Methods Enzymol., 194, 281 (1991))を用いてもよい。これは相同組換えにより改変遺伝子を含むプラスミドDNAを標的遺伝子座に導入した後、形質転換後に生じた内在性の標的遺伝子の一部と形質転換に用いた改変遺伝子の一部とからなる2つの遺伝子の間で起きる自発的な相同組換えにより機能遺伝子が除去され、改変された遺伝子が残された株を選択するという方法である。この選択法においてもURA3遺伝子をマーカーとして用いることにより、5-FOA感受性のUra⁺株の中からURA3遺伝子が自発的な相同組換えにより抜け落ちたUra⁻株の選択は容易である。

【0032】カンジダ・ボイジニを形質転換するための方法としては、プロトプラスト法や酢酸リチウム、電気

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パルス法などを用いることができる。形質転換に用いるカンジダ・ボイジニ株については特に制限されないが、ATCC 48180株や、IFO 10035株等が例示される。また、さらにこの好ましくは少なくともひとつの栄養要求性マーカー遺伝子が欠失した株であり、URA3遺伝子欠失株やLEU2遺伝子欠失株等が例示される。

【0033】(4) 異種遺伝子の発現

異種遺伝子は、転写の読み枠の方向にプロモーター配列、異種タンパク質の構造遺伝子、ターミネーター配列を有する発現ユニットと選択マーカー遺伝子等と共に適当なベクターの中に挿入された異種遺伝子発現ベクターを適当な宿主細胞に導入されることによって行われる。プロテアーゼ遺伝子欠失型カンジダ・ボイジニ株を用いる場合、上記のようにプロテアーゼ遺伝子を破壊し、次に異種タンパク質をコードするDNAで形質転換する。もしくはすでに目的とする異種遺伝子発現ベクターで形質転換された株を後から上記のようにプロテアーゼ欠損株を取得することも可能である。さらに異種遺伝子発現ベクターと上記の改変されたプロテアーゼ遺伝子で同時に形質転換することも可能である。

【0034】組換え異種タンパク質発現のためのプロモーターとしてはカンジダ・ボイジニのアルコールオキシダーゼ遺伝子のプロモーター（特開平5-344895号公報）、ギ酸脱水素酵素遺伝子のプロモーター（国際公開第W0 97/10345号）等が例示される。ターミネーターとしては、カンジダ・ボイジニのアルコールオキシダーゼ遺伝子のターミネーター（特開平5-344895号公報）、ギ酸脱水素酵素遺伝子のターミネーター、アクチン遺伝子のターミネーター（国際公開第W0 97/10345号）等が例示される。なお、異種タンパク質N末端に分泌のためのシグナル配列を連結することにより、異種タンパク質の分泌が可能になる。このような分泌のためのシグナル配列としては本発明で提供されるプロテイナーゼAの分泌シグナルペプチド配列のほか、パン酵母（*S. cerevisiae*）の α 因子の分泌シグナルペプチド配列等が使用できる。

【0035】発現ベクターは宿主染色体DNAに組み込ませたり、宿主細胞内で自己複製可能な自律性複製配列を有するベクターを用いて、プラスミド状態で存在させる。宿主細胞内に存在する異種遺伝子のコピー数は1コピーでも複数であってもよい。このようにして得られた形質転換体を培養し、得られる培養物から精製することにより、目的とする遺伝子発現産物を取得することができる。

【0036】培地としては、メタノール、グリセロール、グルコース等の1種以上の炭素源、及び酵母エキス、トリプトン、肉エキス、ペプトン、カザミノ酸、アンモニウム塩等の1種以上の窒素源に、リン酸、ナトリウム、カリウム、マグネシウム、カルシウム、鉄、銅、

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マンガン、コバルト等の無機塩類を添加し、さらに必要に応じて各種ビタミン、アミノ酸、ヌクレオチド等の微量栄養素を便宜添加したものが挙げられる。

【0037】培地のpHは、5～8の範囲が好ましい。また培養温度は通常15～37℃、好ましくは28℃前後である。培養時間は24～1000時間程度であり、培養は静置、振とう、攪拌、通気下の回分培養又は連続培養により実施することができる。培養終了後、該培養物より遺伝子産物を採取するには、通常のタンパク質精製手段を用いることができる。例えば、形質転換細胞内に生産された場合は、常法により菌体を超音波処理、磨砕処理、加圧破砕等により遺伝子産物を含む粗蛋白質溶液を取得する。必要に応じてプロテアーゼ阻害剤を添加する。培養上清中に生産された場合、培養液そのものから遺伝子産物を回収することができる。得られた溶液をろ過、遠心分離等により固形部分を除去し、粗タンパク質溶液を得る。必要によりプロタミン処理等による核酸の除去を行う。

【0038】粗タンパク質溶液から塩析法、溶媒沈殿法、透析法、限外ろ過法、ゲル電気泳動法、あるいはイオン交換クロマトグラフィー、ゲルろ過クロマトグラフィー、逆相クロマトグラフィー、アフィニティクロマトグラフィー等の精製手法を組み合わせることで、目的タンパク質を分離精製することができる。

【0039】

【実施例】本発明をさらに具体的に説明するために実施例を挙げるが、本発明はこれらにより限定されるものではない。

<実施例1> *Candida boidinii*のプロテイナーゼA遺伝子(PEP4)のクローニング

Candida boidinii ATCC 48180株よりPEP4遺伝子の取得、及びその塩基配列決定を行なった。

【0040】(1-1) プローブの作製

パン酵母 (*Saccharomyces cerevisiae*) (Woolford, C.A. et al., Mol. Cell. Biol. 6, 2500-2510 (1986)) 及びピキア・パストリス (*Pichia pastoris*) (特表平6-506117号公報) 由来のプロテイナーゼAで保存されているアミノ酸配列（一文字表記）：DFAEATSEPG L及びPYDYTTLEVSGSCIに対応する塩基配列のオリゴヌクレオチドを*Candida boidinii*のコードン使用頻度を考慮して以下のように合成した：

PRA5: 5'-GATTTYGCWGAAGCWA
CWTCWGAACCGGGTTT-3'; 及び

PRA3: 5'-ATACAWGAWACTTCYA
AWGTRTAATCRTAWGG-3'.

【0041】プライマーPRA5はアミノ酸配列DFAEATSEPG Lに対応し、プライマーPRA3はアミノ酸配列DFAEATSEPG Lに対応する塩基配列の

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相補鎖の配列である。YPD培地(酵母エキス1%、ペプトン2%、グルコース2%、pH6.0)で培養した *Candida boidinii* ATCC48180株の菌体より、酢酸カリウム法(Methods Enzymol., 65, 404 (1980))によって染色体DNAを調製した。

【0042】*Candida boidinii* 染色体DNAと、プライマーPrA5、PrA3を混合し、Ex Taqポリメラーゼ(宝酒造社)を用いたPCR(94℃で30秒、50℃で1分、72℃で2分)×30サイクルを行った。増幅された約0.6kbのDNA断片を回収し、pT7 Blue T-Vector(ノバジェン社)にクローニングした。Dye primer cycle sequencing FS Ready Reaction Kit(パーキンエルマー社)を用いて、得られたプラスミドの挿入DNA断片の塩基配列を決定したところ、*Saccharomyces cerevisiae* 及び *Pichia pastoris* 由来のPEP4遺伝子のアミノ酸配列と高い相同性を持つアミノ酸配列をコードする塩基配列が認められたので、このDNA断片を *Candida boidinii* のPEP4遺伝子の一部であると断定した。0.6kbの挿入DNA断片は、プラスミドをSalIとEcoRIで切断し、アガロース電気泳動後、回収した。

【0043】(1-2)ライブラリーの作製、及びスクリーニング

Candida boidinii ATCC48180株の染色体DNAを種々の制限酵素で切断し、0.8%アガロースゲル電気泳動を行った。分離したDNAをHybond N+ナイロンメンブレン(アマシャム社)トランスファーした。実施例(1-1)で得られたDNA断片をメガプライマーDNAラベリングシステム(アマシャム社)を用いて放射性標識し、サザンハイブリダイゼーションを行なった。ハイブリダイゼーションは、常法(Molecular cloning 2nd edn., ed. Sambrook, J., et al., Cold Spring Harbor Laboratory U.S.A., 1989)に従って行った。結果、約5.5kbのEcoT22I断片にPEP4遺伝子が存在すると考えられた。そこでそのDNA断片をクローニングすべく、ライブラリーを作製した。*Candida boidinii* の染色体DNAをEcoT22Iで切断し、アガロース電気泳動後、5.5kb付近のDNA断片をゲルから回収した。回収したDNA断片をPstIで切断したpUC118とライゲーションした後、Hanahanの方法(Gene, 10, 63 (1980))で大腸菌DH5α株に形質転換して、ライブラリーを作製した。

【0044】これらライブラリーを前述のDNA断片をプローブとしたコロニーハイブリダイゼーションによりスクリーニングした。得られた陽性クローンの中から、プラスミドpCPRA1及び挿入断片が逆方向であるpCPRA2を保持するクローンを選抜した。

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【0045】(1-3)塩基配列決定

プラスミドpCPRA1の制限酵素地図を作製した(図1)。プラスミドpCPRA1を種々の制限酵素で切断し、サザンハイブリダイゼーションによる解析を行った結果、PEP4遺伝子は図1の約3.5kbのBgIII-EcoT22I領域に存在すると考えられた。本領域の塩基配列の決定を行うために、2.2kbのBgIII-EcoRV断片(図1で下線で示されたBgIIIとEcoRV間の領域)を平滑末端化した後、pUC18のSmaI部位に、1.7kbのHindIII断片(図1で下線で示されたHindIII間の領域)をpBluescript II SK+のHindIII部位に、それぞれ両方向でクローニングした。それぞれのプラスミドより欠失変異体を、double-stranded Nested Deletion Kit(ファルマシア社)を用いて取得した。塩基配列をDye primer cycle sequencing FS Ready Reaction Kit及びDye terminator cycle sequencing FS Ready Reaction Kit(パーキンエルマー社)を用いて決定した。得られた塩基配列をつなぎ合わせることで、配列番号1に示す塩基配列が得られた。

【0046】配列番号1の塩基配列には、1009番目から始まり、2271番目で終わる1263塩基対からなるオープンリーディングフレームが存在する。このオープンリーディングフレームから推定されるアミノ酸配列(配列番号2)と、*Saccharomyces cerevisiae* 及び *Pichia pastoris* 由来のプロテイナーゼAとの相同性を調べたところ、それぞれ75%、68%のアミノ酸が同一であった。シグナル配列切断点予測(von Heijne, Nucleic Acids Res., 14, 4683 (1986))により推定されるシグナルペプチドはメチオニンから22番目のアラニンまでの22アミノ酸であった。

【0047】<実施例2> *Candida boidinii* のプロテイナーゼA遺伝子(PEP4)破壊株の作製

Candida boidinii のURA3遺伝子をマーカーとした形質転換によって、PEP4遺伝子を破壊した。宿主として、*Candida boidinii* ATCC48180株のURA3遺伝子の変異株 *Candida boidinii* SK612株を用いた。*Candida boidinii* SK612株は公知の方法(Sakai Y. et al., J. Bacteriol., 173, 7458 (1991))に従って取得した。

【0048】(2-1)PEP4遺伝子破壊ベクターの作製

図2に示すように、PEP4遺伝子の約2kbのSnaBI-EcoRV領域をURA3遺伝子に置換したプラスミドpDPRA1を作製した。PEP4遺伝子破壊株より、再びウラシル要求株を取得するために、Sakaiらの報告(Sakai Y. et al., J. Bacteriol., 174, 7

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458 (1992))に基づき、構造遺伝子の前後に、反復構造を持ったURA3遺伝子をマーカーとして用いた。

【0049】*Candida boidinii*のURA3遺伝子(Sakai Y. et al., J. Ferment. Bioeng., 73, 255 (1992))を含む2.6 kbのSal I-Pst I断片を、pBluescript II SK-のSal IとPst I部位の間に挿入したpCBU3を作製した。pCBU3をSal Iで切断し、T4 DNAポリメラーゼにより平滑末端処理した後、さらにXba Iで切断して、0.9 kbのURA3遺伝子の5'側を含むDNA断片を単離した。またpCBU3をPst Iで切断し、T4 DNAポリメラーゼにより平滑末端処理した後、さらにKpn Iで切断して得られた2.6 kbのDNA断片と前述の0.9 kbのDNA断片をpUC19のKpn I-Xba Iに挿入し、プラスミドpURPを得た。その結果、pURPをSal I切断して得られる3.5 kbのDNA断片には、URA3構造遺伝子の前後に約0.9 kbの反復配列が存在することになる(図2)。

【0050】pCPRA1と逆向きにPEP4遺伝子が挿入されたpCPRA2をSnaBIとEcoRVで切断し、XhoIリンカー(宝酒造社)を挿入した。得られたプラスミドのXhoI部位にpURPをSal I切断して得られる3.5 kbのDNA断片を挿入し、プラスミドpDPRA1を得た(図2)。

【0051】(2-2) 形質転換

本実施例の(2-1)で得られたpDPRA1をSal Iで切断して、*Candida boidinii* SK612株に酢酸リチウム法(Ito, H. et al., J. Bacteriol., 153, 163 (1983))で形質転換を行った。得られた形質転換体についてその染色体DNAのサザン解析を行うことにより、PEP4遺伝子破壊株をスクリーニングした。すなわち宿主SK612株及び形質転換株の染色体DNAをSal IとNde Iで切断し、pCPRA1をSal IとSnaBI切断してえられる1.7 kbのDNA断片をプローブとしてサザン解析を行った(図3)。図3に示すように宿主SK612株では3.8 kbにバンドが検出されるが、破壊株では5.4 kbの位置にバンドが検出される。

【0052】該破壊株を*Candida boidinii* SK740株と命名した。*Candida boidinii* SK740株をYPD培地で定常期まで培養した後、5-フルオロオロチジン酸(5-FOA)に耐性を示す株を取得した。5-FOA耐性株の取得は実験書(石田功、安東民衛/編、遺伝子発現実験マニュアル、講談社サイエンティフィク、1994)に記載の方法に従った。5-FOA耐性株の染色体DNAをPEP4遺伝子破壊株を取得した際と同様のサザン解析を行うことによって、URA3遺伝子が欠落した株をスクリーニングした。図3に示すようにSK740株では5.

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4 kbの位置に検出されるバンドが、URA3遺伝子が欠落した株では2.8 kbの位置にバンドが検出された。URA3遺伝子が欠落した酵母を*Candida boidinii* SK741株と命名し、平成10年9月1日付で通商産業省工業技術院生命工学工業技術研究所(茨城県、つくば市)にブタペスト条約下に国際寄託され、受託番号FERMBP-6482が与えられた。

【0053】<実施例3> *Candida boidinii*のプロテイナーゼB遺伝子(PRB1)のクローニング

Candida boidinii ATCC48180株よりPRB1遺伝子の取得、及びその塩基配列決定を行なった。

(3-1) プローブの作製

Saccharomyces cerevisiae (Moehle, C.M. et al., Mol. Cell. Biol. 7, 4390-4399 (1987)) 及び*Pichia pastoris* (特表平6-506117号公報)由来のプロテイナーゼBで保存されているアミノ酸配列(一文字表記): GNGHGHGTHCAGT及びATAVLSGTSMIAに対応する塩基配列のオリゴヌクレオチドを*Candida boidinii*のコドン使用頻度を考慮して以下のように合成した:

【0054】PRB5: 5'-GGTAAYGGTCAYGGTACHCAYTGTGCHGGWAC-3'; 及び

PRB3: 5'-GCCATWGAWGTTAGCWGATAARACDGCWGTGDC-3'.

【0055】プライマーPRB5はアミノ酸配列GNGHGHGTHCAGTに対応し、プライマーPRB3はアミノ酸配列ATAVLSGTSMIAに対応する塩基配列の相補鎖の配列である。*Candida boidinii* ATCC48180株の染色体DNAと、プライマーPRB5、PRB3を混合し、Ex Taqポリメラーゼ(宝酒造社)を用いたPCR(94℃で30秒、50℃で1分、72℃で2分)×30サイクルを行った。増幅された約0.5 kbのDNA断片を回収し、pT7Blue T-Vector(ノバジェン社)にクローニングした。Dye primercycle sequencing FS Ready Reaction Kit(パーキンエルマー社)を用いて、得られたプラスミドの挿入DNA断片の塩基配列を決定したところ、*Saccharomyces cerevisiae* 及び*Pichia pastoris*由来のPRB1遺伝子のアミノ酸配列と高い相同性を持つアミノ酸配列をコードする塩基配列が認められたので、このDNA断片を*Candida boidinii*のPRB1遺伝子の一部であると断定した。0.5 kbの挿入DNA断片は、プラスミドをSal IとEcoRIで切断し、アガロース電気泳動後、回収した。

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【0056】(3-2)ライブラリーの作製、及びスクリーニング

Candida boidinii ATCC4818 0株の染色体DNAを種々の制限酵素で切断し、0.8%アガロースゲル電気泳動を行った。分離したDNAをHybond N+ナイロンメンブレン(アマシャム社)トランスファーした。本実施例の(3-1)で得られたDNA断片をメガプライマーDNAラベリングシステム(アマシャム社)を用いて放射性標識し、サザンハイブリダイゼーションを行なった。その結果、PRB1遺伝子は約5.5kbのEcoRI-HindIII断片、約4.5kbのBglII-EcoT22I断片に存在することが示された。次に、約5.5kbのEcoRI-HindIII断片、約4.5kbのBglII-EcoT22I断片をクローニングすべく、ライブラリーを作製した。*Candida boidinii*の染色体DNAをEcoRIとHindIIIで切断し、アガロース電気泳動後、5.5kb付近のDNA断片をゲルから回収した。回収したDNA断片をpUC19のEcoRIとHindIII部位の間に挿入し、EcoRI-HindIIIプラスミドライブラリーを作製した。同様にBglII-EcoT22I断片をpBluescript II SK+のBamHIとPstI部位の間に挿入したBglII-EcoT22Iプラスミドライブラリーを作製した。

【0057】これらライブラリーに上記プローブを用いたコロニーハイブリダイゼーションによるスクリーニングを行った。オートラジオグラフィーによって、EcoRI-HindIIIプラスミドライブラリーからpCPRB1、BglII-EcoT22IプラスミドライブラリーからpCPRB2を保持するクローンが陽性クローンとして選抜された。

【0058】pCPRB1及びpCPRB2の制限酵素地図を作製した(図4)。得られたクローンが*Candida boidinii*のPRB1遺伝子であることを確認すること、及び*Candida boidinii*のPRB1遺伝子のオープンリーディングフレームの位置及び方向を推定することを目的として、前述したゲノミックサザン解析によりプローブがハイブリダイズした最小のDNA断片の約0.7kbのClaI領域の塩基配列を決定した。この塩基配列の決定はpCPRB2より取得した0.7kbのClaI断片を、pBluescript II SK+に挿入して作製したプラスミドを用いて行った。得られた塩基配列(配列番号6)から推定されるアミノ酸配列(配列番号5)と、*Saccharomyces cerevisiae* 及び *Pichia pastoris* 由来のプロテイナーゼBとの相同性を調べたところ、それぞれ76%、77%のアミノ酸が同一であった。この結果より*Candida boidinii*のPRB1遺伝子のオープンリーディング

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グフレームは図4の矢印で示した領域に存在することが推定された。

【0059】<実施例4> *Candida boidinii*のプロテイナーゼB遺伝子(PRB1)破壊株の作製

*Candida boidinii*のURA3遺伝子をマーカーとした形質転換によって、PRB1遺伝子を破壊した。宿主として、実施例2で取得した*Candida boidinii* SK741株を用いた。

(4-1) PRB1遺伝子破壊ベクターの作製

PRB1遺伝子の約0.7kbのClaI領域をURA3遺伝子に置換したプラスミドpDPRB1を次のように作製した。

【0060】pCPRB2をClaIとEcoRIで切断して得られた約2.0kbのDNA断片を、pCPRB1のClaI-EcoRI領域に挿入した。得られたプラスミドをpCPRBΔClaと命名した。pCPRBΔClaをClaIで切断し、T4 DNAポリメラーゼによる平滑末端処理した後、XhoIリンカーを挿入した。得られたプラスミドのXhoI部位に実施例2の(2-1)に記載のpURPをSalI切断して得られる3.5kbのDNA断片を挿入し、プラスミドpDPRB1を得た(図5)。

【0061】(4-2) 形質転換

本実施例の(4-1)で得られたpDPRB1をHincIIとEcoRIで切断して、*Candida boidinii* SK741株に酢酸リチウム法で形質転換を行った。得られた形質転換体についてその染色体DNAのサザン解析を行うことにより、PRB1遺伝子破壊株をスクリーニングした。すなわち宿主SK741株及び形質転換株の染色体DNAをBglIIとHindIIIで切断し、pCPRB1をClaIとBglII切断して得られる1.3kbのDNA断片をプローブとしてサザン解析を行った(図6)。図6に示すように宿主SK741株では3kbの位置に検出されるバンドが、破壊株では5.8kbに検出される。

【0062】該破壊株を*Candida boidinii* SK774株と命名した。*Candida boidinii* SK774株より5-FOA耐性株を取得し、URA3遺伝子が欠落した株をスクリーニングした。スクリーニングはサザン解析によって行った。図6に示すようにSK774株では5.8kbの位置に検出されるバンドが、URA3遺伝子が欠落した株では3.2kbの位置に検出された。該酵母を*Candida boidinii* SK775株と命名した。

【0063】<実施例5> プロテアーゼ欠損株のプロテアーゼ活性の測定

実施例2の(2-2)で得られた*Candida boidinii* SK740 (pep4) 株及び実施例4の(4-2)で得られた*Candida boidi*

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nii SK774 (pep4, prb1) 株、および *Candida boidinii* ATCC48180 株の示すプロテアーゼ活性を測定した。それぞれの株を2mlのYPD培地で、30℃で定常期まで培養した。集菌した菌体を0.2mlの100mM Tris-HClバッファー (pH 7.5) に懸濁し、0.8gのガラスビーズ (0.425-0.6mm、シグマ社) を加え、1分間激しく攪拌後、1分間氷冷するという操作を5回繰り返した。菌体破砕液を4℃、10000回転にて10分間遠心し、上清画分を無細胞抽出液として取得した。プロテインアッセイキット (バイオ・ラッド社) を用いて、無細胞抽出液のタンパク質濃度を測定した。

【0064】無細胞抽出液の酵素活性は、Jonesの総説 (Jones, E. W., Methods Enzymol., 194, 428 (1991)) に従い、プロテイナーゼA活性及びカルボキシペプチダーゼY活性を測定した。すなわち、プロテイナーゼA活性は、25μlの無細胞抽出液、終濃度100mM Glycine-HCl バッファー (pH 3.2)、1%の酸変性ヘモグロビンを含む1mlの反応液中で37℃で測定した。0分後、10分後、20分後、30分後にそれぞれ200μlの反応液を抜き取った後、100μlの1N過塩素酸を加え、10000回転にて10分間遠心した。上清100μlを抜き取り、50μlの0.5M NaOHを加え、本溶液中の遊離ペプチド含量をDCプロテインアッセイキット (バイオ・ラッド社) を用いて測定した。プロテイナーゼA活性は、1分間に1μgのペプチドを遊離する酵素量を1ユニットと定義した。ATCC48180株では無細胞抽出液1mg当たり49.3ユニットのプロテイナーゼA活性が検出されたが、SK740株及びSK774株では活性は検出されなかった。

【0065】カルボキシペプチダーゼY活性は、100μlの無細胞抽出液と500μlのバッファー (100mM Tris-HCl (pH7.5)、1mM CaCl₂) 及び20μlの基質溶液 (ジメチルホルムアミドで溶解させた6mM N-ベンゾイル-L-チロシン-p-ニトロアニリド (シグマ社)) を混合して、よく攪拌した後、37℃で30分間反応した。これに600μlの1.5M 酢酸を加え、反応を停止し、0.22μmのフィルターでろ過し、405nmにおける吸光度を測定した。カルボキシペプチダーゼY活性は、1分間に1nmolのp-ニトロアニリンを遊離する酵素量を1ユニットと定義した。ATCC48180株、SK740株、SK774株が示す無細胞抽出液1mg当たりそれぞれ、0.72ユニット、0.28ユニット、0.05ユニットのカルボキシペプチダーゼY活性が検出され、プロテアーゼ遺伝子欠損により、カルボキシペプチダーゼY活性が大幅に減少することが確認された。

【0066】＜実施例6＞ 異種遺伝子タンパク質の分

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泌生産

プロテアーゼ遺伝子が破壊された *Candida boidinii* 株を用いてウシ由来カテブシンC遺伝子を発現することにより、カテブシンCの分泌量が増大することを確認した。また実施例1の(1-3)で得られたPEP4遺伝子のプレ配列が異種遺伝子タンパク質を分泌させるためのシグナル配列として機能することも確認した。

【0067】(6-1) ウシ由来カテブシンC遺伝子のクローニング

既に報告されているヒト由来カテブシンC遺伝子をPCRにて取得し、得られたDNA断片をプローブとして用いた。ヒトカテブシンC遺伝子の塩基配列 (Patris, A. et al., FEBS Lett., 369, 326 (1995)) に従い、以下のオリゴオリゴヌクレオチドを合成した:

HCat-5: 5'-CAAGGCTTTTGAGAT
TGTGTTGAATGACTAC-3' 及び

HCat-3: 5'-TCTGAGATTGCTGC
TGAAAGTCTACAGTCT-3'.

【0068】鑄型DNAとしてQUICK-Screen Human cDNA Library Panel (Clontech社) を用いた。鑄型DNAと、プライマーHCat-5、HCat-3を混合し、ExTaqポリメラーゼ (宝酒造社) を用いたPCR (94℃で30秒、60℃で30秒、72℃で2分) ×30サイクル) を行った。胎盤由来のライブラリーから増幅された約1.2kbのDNA断片を回収し、pT7 Blue T-Vector (ノバジェン社) にクローニングした。Dye primer cycle sequencing FS Ready Reaction Kit (パーキンエルマー社) を用いて、塩基配列を決定し、ヒト由来カテブシンC遺伝子が挿入されていることを確認した。1.2kbの挿入DNA断片は、プラスミドをSmaIとXbaIで切断し、アガロース電気泳動後、回収し、プローブDNA断片として用いた。

【0069】ウシカテブシンC遺伝子を取得するためのライブラリーとして、ストラタジーン社から購入したBovine Spleen cDNAライブラリーを用いた。添付のプロトコールに従って出現させた約100万の組み換えファージクローンよりブランクハイブリダイゼーションによりスクリーニングした。得られた6個の陽性組み換えファージとライブラリーに添付のヘルパーファージと共に、大腸菌XL1-Blue MRF'株に感染させ37℃で3時間培養し、目的cDNA断片を有するpBluescriptを切り出した。培養液を70℃で20分間処理した上清液を大腸菌SOLR™株に感染させ、組み換えプラスミドDNAを有する大腸菌をアンピシリン耐性により選抜した。

【0070】6個の組み換えプラスミドをDye primer cycle sequencing FS Ready Reaction Kit (パーキンエルマー社) を用いて、5'末端側、3'末端側の塩基配列

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を決定した結果、最も長いcDNA断片を有するクローンとしてpBC20-2を選抜した。Dye terminator cycle sequencing FS Ready Reaction Kit (パーキンエルマー社)を用いてpBC20-2の挿入DNA断片の塩基配列を決定し、配列番号7に示す塩基配列を得た。得られた塩基配列より配列番号8に示すアミノ酸配列が得られた。この配列とヒトカテプシンCのアミノ酸配列を比較したところ、89%のアミノ酸が同一であった。プロ領域のN末端は配列番号8の20番目のアスパラギン酸、成熟領域のN末端は配列番号8の226番目のロイシンであると考えられた。pBC20-2には開始メチオニンをコードする配列が含まれておらず、ブレ領域の一部は欠損していると考えられた。

【0071】(6-2)ウシカテプシンC発現プラスミドの構築

本実施例の(6-1)で得られたウシカテプシンCを*Candida boidinii*を用いて分泌発現させるために、実施例1の(1-3)で得られたプロテイナーゼAのブレ領域(配列番号4)をウシカテプシンCのプロ領域-成熟領域のN末端側に連結した。またウシカテプシンCのプロ領域-成熟領域の塩基配列は、*Candida boidinii*において使用頻度の高いコドンを用いた塩基配列配列に変換した。さらに構造遺伝子の翻訳開始コドン(ATG)の5'上流側及び翻訳終止コドン(TAA)の3'下流側にNotI認識部位が形成されるように設計した(配列番号9)。設計したDNAは図7に示す方法で、PCRを用いて合成した。図7中の各プライマーの塩基配列を以下に示す：

A1F: 5'-GTACATATCCAGATCTAT
TAGGTA CTTGGGTCTTTCAAGTTGG
TTCTTCTG GTTCACAAAGAGATGTT
AATTGTTCTGTTATGGGTCCTCCAG
AGAAGAAAGTTGTCGTTCACTTAAA
GAAACTTG-3' ;

A1R: 5'-GCAAACCATTTTATAATCA
TTCAAGACAATTTTCGAAACCTTGAT
TATAGATAATAGTGAAATGACCAGA
ATTACCAAAATCATCATAAGCAGTA
TCAAGTTTCTTTAAGTGAACGACAA
CTTTCTTC-3' ;

A2F: 5'-GGGGGGGCGGCCGCGCATGAA
GTTTACAAATTCCTTTTTTCTGTGCT
TTCTCTATCTTAGCTGCTACTACTT
TAGTTGATGCTGATACTCCAGCTAA
TTGTACATATCCAGATCTATTAGGT
ACTTGGG-3' ;

A2R: 5'-CCCCCACTAGTCCTAGGA
CATCATGAACCCAACCTGTCATAGT
TTCATGACAATAAGAAGTAACTTTA
CCACCTTCTTCTTTATATTTAAAGA

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AAGCAAACCATTTTATAATCATTCAA
GACAATTTTCG-3' ;

B1F: 5'-CGTTAATACTGCTAGATT
AGCTGGTTT TAGAAGAAACATACTCT
AATAGATTATATCGTTATAATCATG
ATTTTCGTCAAAGCTATTAATGCTAT
TCAAAAATCTTGGAC-3' ;

B1R: 5'-TACGAGAATGACCACCAC
CTCTTCTAATCATTCTTTAAGAGT
TAATGTTTTCATATTCATATAAGGA
GCAGCAGTCCAAGATTTTTTGAATAG
CATTAATAGCTTTG-3' ;

B2F: 5'-GGGGGGGCGGCCGCGGGGC
CTAGGTAGAAATTGGGCTTGTTTCA
CTGGTAGAAAGACTGGTAATACTTCT
TGAAAATGTTAACGTTAATACTGCT
AGATTAGCTGGTTT TAGAAG-3' ;

B2R: 5'-CCCCCACTAGTAGGTAAG
TGTAAGATTTTCTTCTGAATTTTCAG
CAGTAATAGGTGCAGGTTTAGGTCT
AGGTATTCTACGAGAATGACCACCA
CCTCTTCTAATCA-3' ;

C1F: 5'-TTGCTTCTATGGGTATGA
TGGAAGCTAGAAATTAGAATTTTGAC
TAATAATACTCAAACCTCCTATCTTA
TCTCCACAAGAAGTTGTCTCTTGTT
CTCAATATGCTCAAGGTTGTGAAGG
TG-3' ;

C1R: 5'-ATGGAGAATCAGTACCAG
TATATGGAAAACAATCTTCTTCAAC
TAGACCAAAGTCCTGAGCATATTTA
CCAGCAATTAAGTATGGGAAACCCAC
CTTCACAACCTTGAGCATATTGAGA
AC-3' ;

C2F: 5'-GGGGGACTAGTTGGGATT
GGAGAAATGTTTCATGGTATTAACCTT
TGTTACTCCTGTTAGAAATCAAGGT
TCATGTGGTTCTTGTACTCATTG
CTTCTATGGGTATGATGGAAGCTAG

AATTAGAATTTTGAC-3' ;

C2R: 5'-CCCCCAAGCTTCATTACA
ACCACCATAGAAACCACCAACATAA
TGATATTTCAGAAGAGTAATATCTGA
AACAACTTCTTTCAATCTACATGG
AGAATCAGTACCAGTATATGGAAAA
C-3' ;

D1F: 5'-ATTATAGAAAAGGTGTTT
ATCATCACACTGGTTT TAGAGATCC
ATTTAATCCATTTGAGCTCACTAAT
CATGCTGTCTTATTAGTTGGTTATG

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GTACTGATGCTGCTTCTG-3';
 D1R: 5'-GTACCTCTTCTAATTCTA
 AAGTAACCATTTTTACCCCCAAGAAG
 TACCCCATGAGTTCTTAACAATCCA
 ATAATCTAAACCAGAAGCAGCATCA
 GTACCATAACCAACTAATAAG-3';
 D2F: 5'-GGGGGAAGCTTTGATGAA
 ATTAGAATTAGTTCATCAAGGTCCT
 ATGGCTGTTGCTTTTGAAGTCTATG
 ATGATTTCTTACATTATAGAAAAGG
 TGTTTATCATCACACTG-3'; 及び
 D2R: 5'-CCCCCTCGAGGCGGCCG
 CTTATAATTTAGGAATAGGAGTAGC
 AGCTAAAGCAATAGATTCAATAGCA
 CATTTCATCAGTACCTCTTCTAATTC
 TAAAGTAACCATTTTTTC-3'。

【0072】領域Aは、まずプライマーA1FとA1Rを混合し、Ex Taqポリメラーゼ（宝酒造社）を用いたPCR（94℃で30秒、60℃で1分、72℃で30秒）×20サイクル）を行い、2本鎖DNAを合成した。反応終了後、フェノール／クロロホルム抽出、エタノール沈殿を行い、PCR反応液の2分の1容量（25μl）のTEバッファーに溶解した。この溶液2μlとプライマーA2FとA2Rを混合し、Ex Taqポリメラーゼ（宝酒造社）を用いたPCR（94℃で30秒、60℃で1分、72℃で30秒）×20サイクル）を行った。増幅されたDNA断片を回収し、Not IとSpe Iで切断した後、pBluescript II KS+のNot I-Spe I間に挿入した。Dye primer cycle sequencing FS Ready Reaction Kit（パーキンエルマー社）を用いて、領域Aが正しく合成されていることを確認した。得られたプラスミドをpCT-Aと命名した。

【0073】領域B、C、Dについても図7に示すプライマーを用いて領域Aと同様の方法で合成し、それぞれpBluescript II KS+に挿入したプラスミドpCT-B、pCT-C、pCT-Dを得た。pCT-Aから切り出したNot I-Sty I断片をpCT-BのNot I-Sty I間に挿入したプラスミドpCT-AB、pCT-Cから切り出したSpe I-Hind III断片をpCT-DのSpe I-Hind III間に挿入したプラスミドpCT-CDを作製した。pCT-CDから切り出したSpe I-Xho I断片をpCT-ABのSpe I-Xho I間に挿入し、プラスミドpCTC-S1を作製した。

【0074】pCBU3から切り出したCandida boidinii URA遺伝子を含む2.6kbのSal I-Pst I断片とpFdhPT (WO97/10345) から切り出したCandida boidinii ギ酸脱水素酵素遺伝子プロモーター／ターミネ

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ーター領域を含む2.1kbのKpn I-Eco T22 I断片をpUC19のKpn I-Sal I間に挿入し、マーカー遺伝子がURA3遺伝子で、ギ酸脱水素酵素遺伝子プロモーター／ターミネーターによる異種遺伝子発現プラスミドpFexU3を作製した（図8）。pCTC-S1から切り出したカテプシンC遺伝子を含むNot I断片をpFexU3のNot I部位に挿入し、ウシカテプシンC発現プラスミドpECTC-S1を作製した（図8）。

【0075】（6-3）形質転換

本実施例の（6-2）で得たプラスミドpECTC-S1をBamHIで切断し、Candida boidinii SK612株、SK741株に形質転換した。得られた形質転換体のコロニーを各宿主株につき10個拾い、培地中に分泌されるカテプシンC活性を測定した。まず、GLYS培地（グリセロール3%、Yeast Nitrogen Base 0.67%、Yeast Extract 0.5%を含むpH5.5の培地）中で30℃にて、48時間振とう培養した。3000回転、5分間の遠心で集菌した菌体をGLYS培地と等量のMYS（メタノール1.5%、Yeast Nitrogen Base 0.67%、Yeast Extract 0.5%を含むpH5.5の培地）に懸濁し、さらに30℃にて、48時間振とう培養した。培養後、3000回転、5分間の遠心によって取得した培養上清をマイクロコン-30（アミコン社）を用いて50倍濃縮し、以下に示す方法でカテプシンC活性を測定した。

【0076】2μlの濃縮培養上清と、200μlのバッファー（50mMクエン酸-クエン酸ナトリウムバッファー（pH5.0）、10mM NaCl、1mMβ-メルカプトエタノール、及び基質の4mM Glycyl-L-phenylalanine-p-nitroanilide（シグマ社、ジメチルホルムアミドで200mMに溶解させたものを希釈））を混合した後、37℃で2～10時間放置し、405nmにおける吸光度を測定した。標準品としてベーリンガー社から購入したウシカテプシンCの16、8、4、2、1、0.5μg/ml溶液を調製し、これを試料として作製した標準曲線から、各サンプルのカテプシンC活性を算出した。各形質転換株が示したカテプシンC生産量を図9に示す。図9に示すようにプロテイナーゼ遺伝子が破壊されたCandida boidinii株を宿主として用いた場合の方が、カテプシンC生産性に優れていた。

【0077】

【発明の効果】本発明によりプロテアーゼ（又はタンパク質分解）活性の減少したCandida boidinii株が提供され、この酵母を宿主とした発現系において目的タンパク質の分解を防ぐことにより該タンパク質の収率を向上させることができる。

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【0078】

【配列表】

SEQUENCE LISTING

<110> Kirin Brewery Company, Limited

<120> Candida boidinii strains and use thereof as hosts for preparing heterologous proteins

<130> P98-0444

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<213> Candida boidinii

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【図面の簡単な説明】

【図1】この図はプロテイナーゼA遺伝子を含むプラスミドpCPRA1の制限酵素地図を示す。

【図2】この図はプロテイナーゼA遺伝子破壊プラスミドpDPRA1の構築手順を示す。

【図3】この図はCandida boidinii SK612株、SK740株、SK741株のPEP4遺伝子座の制限酵素地図を示す。

【図4】この図はプロテイナーゼB遺伝子及び、プラスミドpCPRB1、pCPRB2の制限酵素地図を示す。

す。

【図5】この図はプロテイナーゼB遺伝子破壊プラスミドpDPRB1の構造を示す。

【図6】この図は、*Candida boidinii* SK741株、SK774株、SK775のPRB1遺伝子座の制限酵素地図を示す。

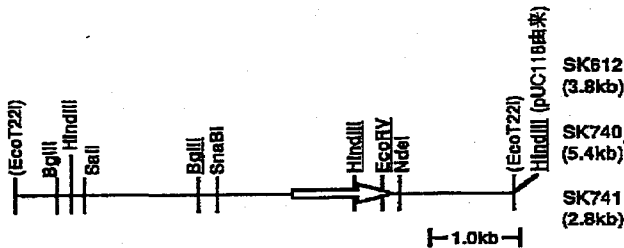
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順を示す。

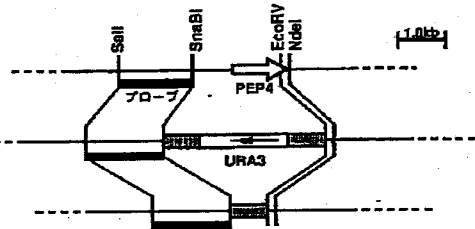
【図8】この図は、プラスミドpECTC-S1の構築手順を示す。

【図9】この図は、*Candida boidinii* SK612株及びSK741株を宿主としたカテプシンC発現株の培地上清のカテプシンC活性を示す。

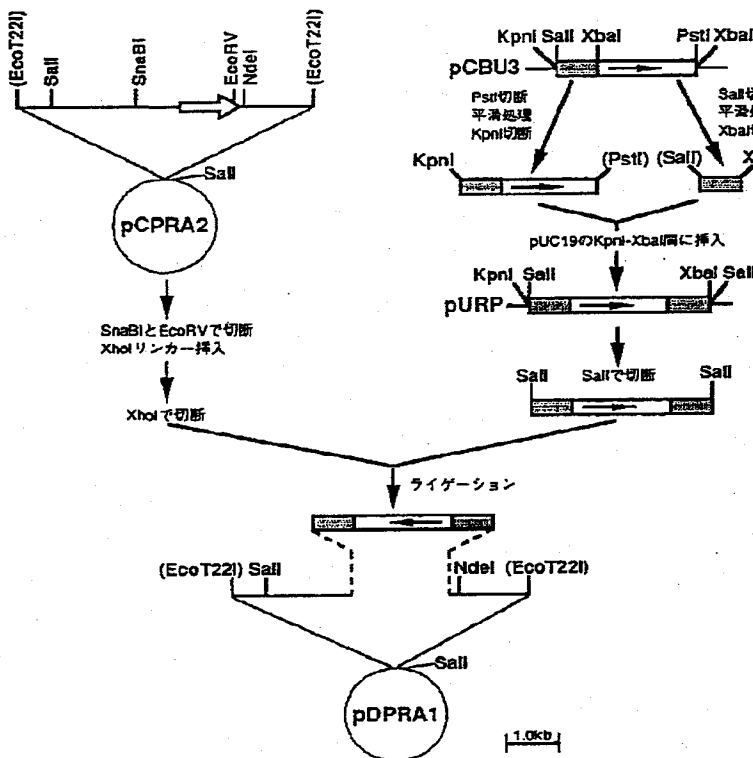
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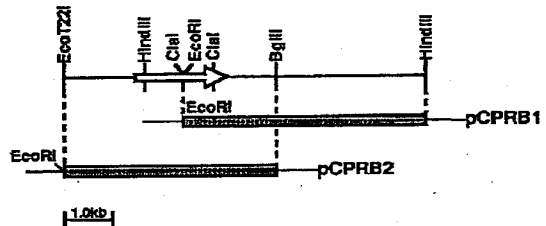
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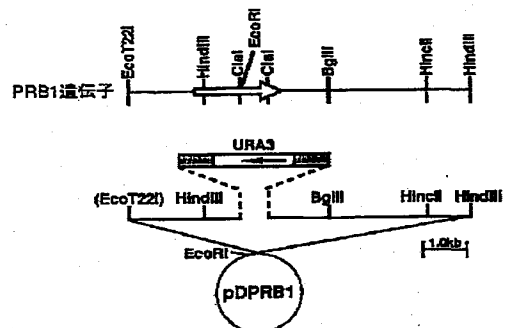
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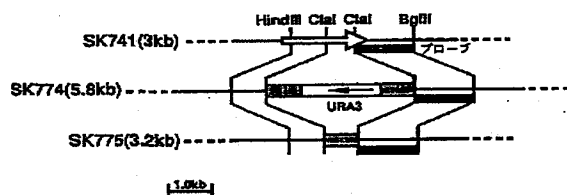
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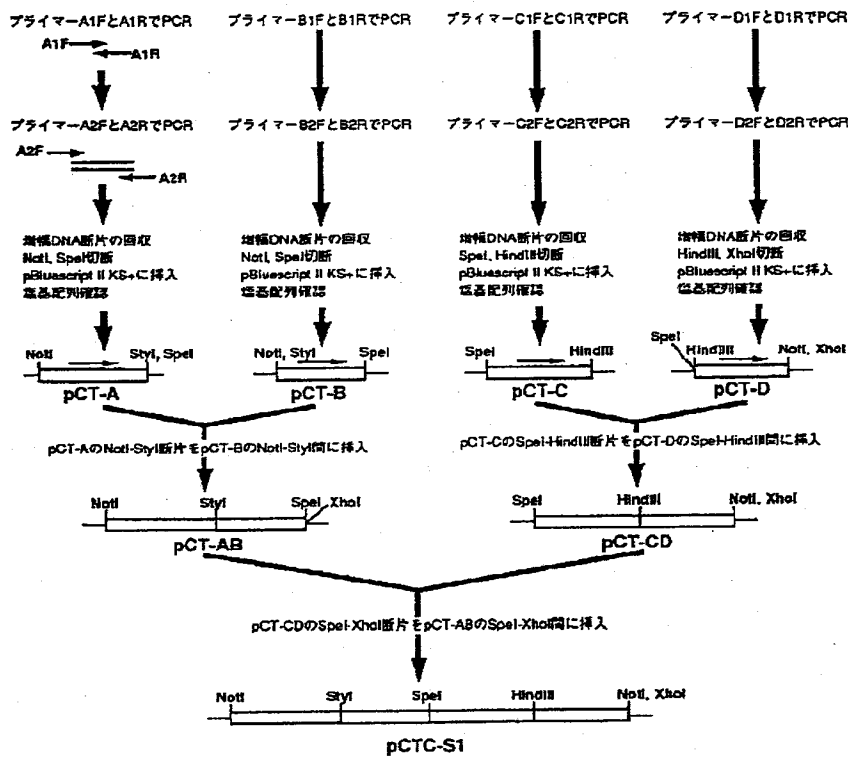
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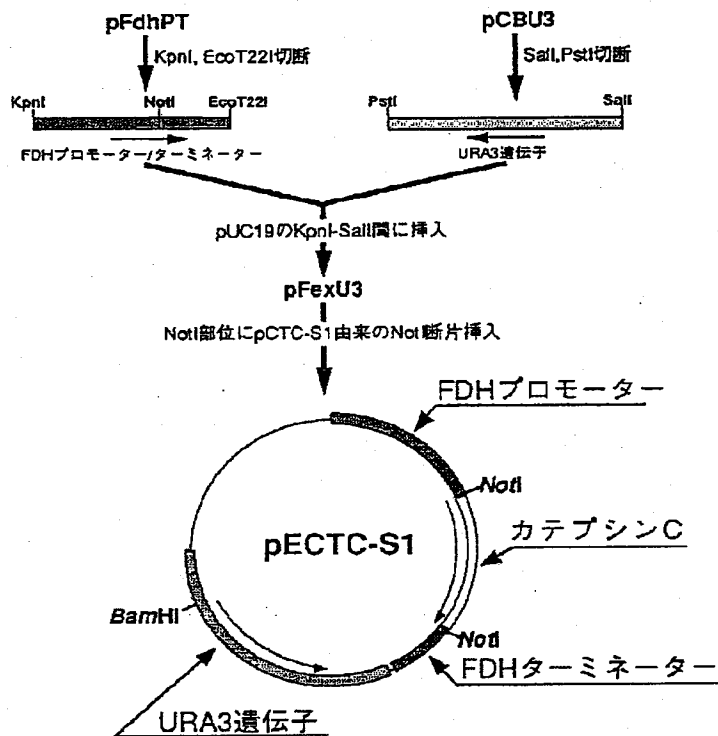
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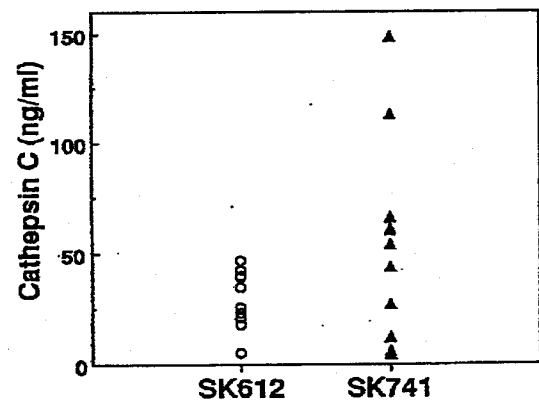
【図 7】



【図 8】



【図 9】



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C 1 2 P 21/02		C 1 2 P 21/02	C
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 4B050 CC03 DD11 EE10 LL05
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 4H045 AA10 AA20 BA10 CA40 DA89
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 C12P 21/02
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(21)Application number : 10-251526

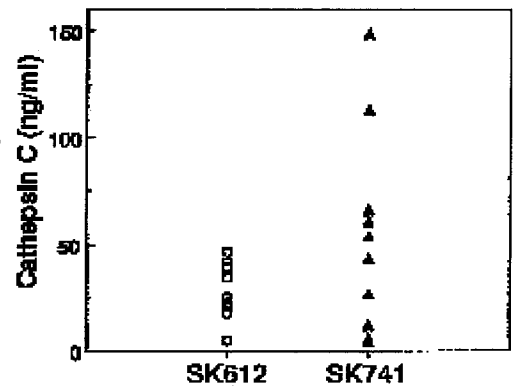
(71)Applicant : KIRIN BREWERY CO LTD

(22)Date of filing : 04.09.1998

(72)Inventor : YONEDA TOSHIHIRO
 KONDO KEIJI**(54) CANDIDA BOIDINII STRAIN HAVING DECREASED PROTEASE ACTIVITY AND ITS USE AS HOST CELL FOR PRODUCTION OF HETEROLOGOUS PROTEIN****(57)Abstract:**

PROBLEM TO BE SOLVED: To obtain the subject new strain consisting of a Candida boidinii strain having decreased protease activity, enabling high expression of heterologous genes, and usable e.g. as host cells capable of producing useful heterologous proteins, such as cathepsin C, in a high yield.

SOLUTION: This strain is a new Candida boidinii strain having decreased protease activity (e.g. Candida boidinii SK 741 strain), enables high expression of heterologous genes, and is capable of producing useful heterologous proteins, such as cathepsin C, in high yield. This new strain is obtained by separating chromosomal DNAs from Candida boidinii ATCC 48180 strain, preparing a genomic library by using the chromosomal DNAs, screening the library by a probe to yield a proteinase A gene, elucidating the base sequence of the proteinase A gene, constituting a protease gene-disruptive plasmid, preparing a transformant by using the plasmid to acquire a protease gene-deficient strain.

**LEGAL STATUS**

[Date of request for examination]

10.08.2005

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[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

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CLAIMS

- [Claim(s)]
- [Claim 1] The Candida BOIJINI (Candidaboidinii) stock with which protease activity fell.
- [Claim 2] The Candida BOIJINI stock according to claim 1 lost by Proteinase A, proteinase B, or the protease activity of the both.
- [Claim 3] The Candida BOIJINI stock according to claim 2 which are 740 shares of Candida BOIJINI [774 shares of] SK, SK [741 shares of], and SK, or 775 shares of SK.
- [Claim 4] The proteinic manufacture approach which carries out a transformation by the expression vector containing the gene which carries out the code of the useful different-species protein for the Candida BOIJINI stock according to claim 1 to 3, and includes collecting the different-species protein cultivated and generated in the suitable culture medium.
- [Claim 5] The approach according to claim 4 different-species protein is cathepsin C.
- [Claim 6] The approach containing DNA to which an expression vector adjoins the five prime end of the gene which carries out the code of the different-species protein, and carries out the code of the secretion transit peptide array according to claim 4 or 5.
- [Claim 7] The approach according to claim 6 a secretion transit peptide array is the thing of the protease protein origin.
- [Claim 8] The approach according to claim 7 a secretion transit peptide array consists of an amino acid sequence shown in the array number 4.
- [Claim 9] The proteinase A of the Candida BOIJINI stock origin which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added so that it may have at least 80% of homology in the amino acid sequence of the place [23rd] - the 420th place shown in the array number 2, or its array, and has protease activity, or its derivative.
- [Claim 10] DNA which carries out the code of the proteinase A of the Candida BOIJINI stock origin according to claim 9, or its derivative.
- [Claim 11] The precursor proteinase A of the Candida BOIJINI stock origin in which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added so that it may have at least 80% of homology in the amino acid sequence shown in the array number 2, or its array, or its derivative.
- [Claim 12] DNA which carries out the code of the precursor proteinase A of the Candida BOIJINI stock origin according to claim 11, or its derivative.
- [Claim 13] DNA according to claim 12 which has the base sequence shown in the array number 3.
- [Claim 14] The proteinase B or its derivative of the Candida BOIJINI stock origin which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added so that it may have at least 80% of homology in the amino acid sequence shown in the array number 5, or its array, and has protease activity.
- [Claim 15] DNA which carries out the code of the proteinase B of the Candida BOIJINI stock origin according to claim 14, or its derivative.
- [Claim 16] DNA according to claim 15 which has the base sequence shown in the array number 6.
- [Claim 17] Secretion transit peptide of the Candida BOIJINI origin proteinase A which consists of an amino acid sequence shown in the array number 4.
- [Claim 18] 741 shares of Candida BOIJINI SK.
- [Claim 19] The manufacture approach of cathepsin C which carries out a transformation by the expression vector containing the gene which carries out the code of the cathepsin C for 741 shares of Candida BOIJINI SK, and includes collecting the cathepsin C cultivated and generated in the suitable culture medium.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Field of the Invention] This invention relates to the manufacturing method of different-species protein using the Candida BOIJINI stock and this Candida BOIJINI stock which have DNA which changed the protease gene of Candida BOIJINI (Candida boidinii), and this protease gene as a host. If the protein manifestation system which makes this Candida BOIJINI stock a host is used, object different-species protein is producible with sufficient yield. Moreover, this invention relates to the manufacturing method of the different-species protein using the secretion manifestation system of different-species protein which uses this transit peptide for the secretion manifestation of the different-species protein which makes Candida BOIJINI a host about useful transit peptide, and this secretion manifestation system.

[0002]

[Description of the Prior Art] Methanol-utilizing yeast Candida BOIJINI has been developed as an effective host of a different-species protein manifestation system in recent years. If the alcohol oxidase which exists in a methanol utilization path, dihydroxyacetone synthase, and formic-acid dehydrogenase are cultivated under methanol existence, remarkable production is carried out and the manifestation approach of a heterologous gene using the control region of those genes is studied (JP,5-344895,A, international disclosure the WO No. 97/10345 etc.). However, the object product may be disassembled by the protease of the host origin when producing different-species protein by gene recombination. In such a case, the volume of object protein decreases and purification of object protein becomes difficult by mixing of a proteolysis product.

[0003] In order to avoid the problem of disassembly of the object protein produced by gene recombination, the culture approach which checks the protease activity which disassembles object protein has been used. For example, it is possible to check a protease operation by adjusting pH of the culture medium which cultivates recombinant. However, this approach will affect growth of the host yeast which discovers different-species protein of a certain kind, and is effective only for disassembly of the protein in the outside of a cell.

[0004] By on the other hand using the stock which inactivated Proteinase A and proteinase B in yeast Saccharomyces cerevisiae and Pichia pastoris as a protease deficit stock the example of having made the inside of a biomass and the protein production outside a biomass increase is shown (the Patent Publication Heisei No. 506117 [six to] official report —) Weis, H.M. et al., FEBS Lett., 377,451 (1995), Inoue, K. et al., Plant Cell Physiol., 38 (3), 366 (1997).

[0005] Proteinase A and proteinase B are the proteases which carry out localization to a vacuole, and the code is carried out by PEP4 gene and PRB1 gene, respectively. According to research of yeast Saccharomyces cerevisiae, Proteinase A and proteinase B activate another proteases, such as themselves and Carboxypeptidase Y, (12 vandenHazel, H.B. et al., YEAST, 1 (1996)). By the way, although it was not known at all about using a protease deficit stock in order to raise a protein volume when making a heterologous gene discover using Candida BOIJINI, there was a trouble as shown in the following which resists such remembrance in this contractor.

[0006] Saccharomyces cerevisiae and Pichia pastoris differ from Candida BOIJINI intrinsically in mycology, and many metabolic turnover physiological difference exists. So, it is guessed easily that proteolysis devices differ. The knowledge about the proteolysis activity which exists in Candida BOIJINI for the moment does not exist at all. Without this invention persons this time Saccharomyces cerevisiae, Although it was found out in Pichia pastoris that activity remains about 40% in Candida BOIJINI to which the carboxypeptidase Y which loses activity thoroughly suffered a loss in the proteinase A gene by the proteinase A genetic defect It is shown that this example also differs from the thing of other two yeast of the above [the proteolysis device of Candida BOIJINI].

[0007] Acquisition of a protease deficit stock is acquired by screening of a variant, and gene disruption. In order to screen a variant, it must analyze about a huge number of variants, and in Candida BOIJINI which does not have sporulation ability unlike Saccharomyces cerevisiae and Pichia pastoris, it is impossible to analyze whether variation was introduced only into the target gene by crossing. The revertant which returns to the condition before the characteristic which furthermore varied varying may also happen. On the other hand, since it is possible to make only the target gene suffer a loss as for a gene destructive procedure, it is effective technique, but in order to perform gene disruption, a host's object gene field must be gained. However, such a protease about Candida BOIJINI and the knowledge about the gene are not acquired at all to current. Therefore, improvement in the productivity in the manifestation system using the protease deficit stock of Candida BOIJINI was not considered at all until now.

[0008]

[Problem(s) to be Solved by the Invention] This invention aims at offering the protease originating in Candida BOIJINI, DNA which has the base sequence which carries out the code of the protease, and the Candida BOIJINI stock in which the protease gene carried out deletion. Moreover, it aims at offering the secretory production approach of different-species protein of having used transit peptide useful in a yeast secretion manifestation system, and its transit peptide for the DNA array which carries out the code of the secretion transit peptide originating in the protease protein.

[0009]

[Means for Solving the Problem] this invention persons came to complete this invention by analyzing the protease gene of the methanol-utilizing yeast Candida boidinii, and attaining the high manifestation of a heterologous gene using a protease deletion Candida BOIJINI stock, as a result of inquiring wholeheartedly that the efficient manifestation of a heterologous gene should be attained. This invention offers the Candida BOIJINI stock with which protease activity fell. Here, as for a protease, it is desirable that it is what participates in disassembly of the different-species protein generated by the manifestation with a recombination technique. On the

whole by making at least one activity of the kind of protease lose, protease activity can be reduced.

[0010] In the embodiment of this invention, this invention offers the Candida BOIJINI stock lost by Proteinase A, proteinase B, or the protease activity of the both. More specifically, such strain is 740 shares of Candida BOIJINI [774 shares of] SK, SK [741 shares of], and SK, or 775 shares of SK. The transformation of this invention is carried out by the expression vector which contains the gene which carries out the code of the useful different-species protein for this above-mentioned Candida BOIJINI stock again, and the proteinic manufacture approach including collecting the different-species protein cultivated and generated in the suitable culture medium is offered.

[0011] In the embodiment of this invention, a transformation is carried out by the expression vector which contains in this invention the gene which carries out the code of the cathepsin C for 741 shares of Candida BOIJINI SK, and the manufacture approach including collecting the cathepsin C cultivated and generated in the suitable culture medium of cathepsin C is included. An expression vector can contain DNA which adjoins the five prime end of the gene which carries out the code of the different-species protein, and carries out the code of the secretion transit peptide array. Transit peptide has the role which conveys the precursor protein compounded with the Golgi body to the film, and will be cut by the signal peptidase, and maturation protein will be secreted out of a cell as a result. A secretion transit peptide array consists of a thing of the protease protein origin, and an amino acid sequence more specifically shown in the array number 4 of the proteinase A origin of Candida BOIJINI in the embodiment of this invention.

[0012] This invention offers further the proteinase A of the Candida BOIJINI stock origin which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added so that it may have at least 95% of homology more preferably, and has protease activity, or its derivative at least 90% preferably at least 80% in the amino acid sequence of the place [23rd] – the 420th place shown in the array number 2, or its array. This invention also offers DNA which carries out the code of the proteinase A of the above-mentioned Candida BOIJINI stock origin, or its derivative further again.

[0013] In the amino acid sequence shown in the array number 2, or its array, at least 80%, preferably, this invention offers the precursor proteinase A of the Candida BOIJINI stock origin in which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added, or its derivative at least 90% again so that it may have at least 95% of homology more preferably. This invention also offers DNA which carries out the code of the precursor proteinase A of the further above-mentioned Candida BOIJINI stock origin, or its derivative. Specifically, this DNA can have the base sequence shown in the array number 3.

[0014] This invention offers preferably the proteinase B of the Candida BOIJINI stock origin which one or more amino acid has deletion and the amino acid sequence permuted, inserted and/or added so that it may have at least 95% of homology more preferably, and has protease activity, or its derivative at least 90% at least 80% again in the amino acid sequence shown in the array number 5, or its array. This invention also offers DNA which carries out the code of the proteinase B of the further above-mentioned Candida BOIJINI stock origin, or its derivative. Specifically, this DNA has the base sequence shown in the array number 6.

[0015] DNA which carries out the code of the proteinases A and B of the Candida BOIJINI stock or the precursor proteinases A and B, and them Baker's yeast (*Saccharomyces cerevisiae*) or the proteinase A of *Pichia pastoris* (*Pichia pastoris*) Although it has the similarity on PEP4 and PRB1 gene which carry out the code of B and them, and an array The proteinases A and PEP4 of *Saccharomyces cerevisiae* or *Pichia pastoris* and PRB1 gene have homology lower than both 80%, and the amino acid differs from a DNA array intrinsically. So, the above-mentioned "derivative" in this invention means that variation, such as a permutation, deletion, insertion, or addition, may be included as long as the protease activity made into the object is acquired, and/or as long as it has at least 95% of homology more preferably at least 90% at least 80% with those arrays in the array shown in the array numbers 2 and 3 or the array numbers 5 and 6. Moreover, a DNA array with sufficient homology to destroy the homologous gene on DNA with the base sequence of the arbitration which carries out the code of the same amino acid sequence to the amino acid sequence shown in the array number 2 or 5 intrinsically, or a chromosome is also included in the derivative of this invention. For example, even if the 6th "the g" of the base sequence shown by the array number 3 is permuted by "a", as long as the protease activity made into the object of this invention is acquired, it means that this permuted array is also included in this invention. It is also possible to express to have the array which includes substantially the amino acid sequence or base sequence the derivative of this invention is indicated to be to the array numbers 2, 3, and 5 or 6 in both protein and DNA at this point. This invention offers the secretion transit peptide of the Candida BOIJINI origin proteinase A which consists of an amino acid sequence shown in the array number 4 again.

[0016]

[Embodiment of the Invention] The protease deletion Candida BOIJINI stock to which proteolysis activity fell remarkably to the old stock by this invention when the base sequence which carries out the code of the protease of Candida BOIJINI had [production of this protease] this alteration DNA in DNA (a permutation, deletion, insertion, addition, etc.) changed so that it might be controlled at least, DNA by which the transformation marker gene was inserted in the base sequence which carries out the code of this protease preferably, and a list is offered.

[0017] It not only does not produce the wild type proteinase A at all, but as an example of such a stock, it is the Candida BOIJINI stock permuted with PEP4 gene with which PEP4 gene which carries out the code of the proteinase A of a wild type was changed as mentioned above, and, originally protease activity activated by Proteinase A, such as Carboxypeptidase Y and proteinase B, is remarkably controlled on this stock. Moreover, the Candida BOIJINI stock permuted with PRB1 gene with which PRB1 gene which carries out the code of the proteinase B in addition to PEP4 gene was changed as mentioned above can also be created, and it is expected on a PEP4 deletion stock in such a duplex variant that the proteinase-B activity it is supposed that activity remains slightly will not be detected at all, either. The Candida BOIJINI stock and PEP4 deletion stock with which protease activity fell, and PEP4 and a PRB1 deletion stock have the description of holding the proliferation potential force equivalent to a wild strain under the culture condition which used the nutrition culture medium. As for this, the existence of these genes means not affecting growth of Candida BOIJINI under nutrition conditions. Therefore, the yeast with which the protease activity by this invention was controlled is the host who excelled for different-species protein production. Especially the yeast concerned can produce the different-species protein of protease susceptibility efficiently.

[0018] The transformant obtained by carrying out a transformation by the expression vector which contains the gene (namely, heterologous gene) which carries out the code of the different-species protein for this further above-mentioned Candida BOIJINI stock by this invention is cultivated, and the manufacture approach of different-species protein including collecting object protein from a biomass or a culture supernatant is offered. Although a heterologous gene means the gene of arbitration set as the object of a manifestation here, for example, cathepsin C, an epidermal growth factor (EGF), insulin like growth factor 1 (IGF-1), a human serum

albumin, erithropoietin (EPO), SUROMBOPOI ethyne (TPO), etc. are mentioned, it is not limited to these. Moreover, a heterologous gene may be obtained by what kind of technique.

[0019] Moreover, an expression vector can adjoin and (namely, furan king) contain DNA which carries out the code of the transit peptide array for secretion of different-species protein in the five prime end of the gene which carries out the code of this different-species protein. It makes it possible to make this secrete the different-species protein generated by manifestation out of a cell. As this secretion transit peptide, the transit peptide of the proteinase A of the signal sequence which cathepsin C originally has, the secretion transit peptide array of alpha factor of baker's yeast, and Candida BOIJINI can be used, for example.

[0020] In the embodiment of this invention, the gene which carries out the code of the cathepsin C which is the JIPEPUCHIJIRU protease of the cow origin as a heterologous gene is illustrated. This enzyme is a protease which disassembles [each] two amino acid from a proteinic amino terminal, and is a useful enzyme on industry. It is characterized as this new gene being the amino acid sequence shown in the array number 8, and protein which has the same amino acid sequence substantially. Cathepsin C becomes the maturation protein which it is discovered as an inactive precursor (pre pro object), and the pre peptide field which functions as a secretion signal by subsequent processing is cut, a pro peptide field is cut continuously, and has activity. A pro peptide field is a peptide fragment generally contained in a proteinic inactive precursor, cutting clearance of this ** PUCHIDO is carried out by the specific protease etc. from an inactive precursor, and activity peculiar to the protein is shown. Therefore, the pro peptide array of cow cathepsin C and the Pori ** PUCHIDO array which constitutes the maturation protein following it are required for activation in the secretion process of this enzyme. For this reason, it is required for the secretion manifestation in the yeast of this enzyme to add the signal sequence for secretion to the amino terminal of the pro peptide of cathepsin C. the thing of the above-mentioned instantiation as this secretion transit peptide -- the transit peptide of the proteinase A of Candida BOIJINI can be used preferably. Moreover, for the manifestation of this protein, especially the above-mentioned protease genetic defect stock was useful.

[0021] Therefore, the manufacture approach of cow origin cathepsin C using the Candida BOIJINI stock with which protease activity fell to the Candida BOIJINI stock which produces cathepsin C, the Candida BOIJINI stock with which protease activity fell suitably, and the list is also included by this invention. Hereafter, this invention is further explained to a detail.

[0022] In order to solve the above-mentioned technical problem, this invention persons solved the proteinase A of (1) Candida BOIJINI, and the base sequence of a proteinase-B gene, built (2) protease gene disruption plasmid, produced the transformant using (3) book plasmid, and acquired the protease deletion Candida BOIJINI stock. When a heterologous gene is made to discover by making (4) protease deletion Candida BOIJINI stock into a host furthermore, it checks excelling the time of the volume making a wild strain a host, and comes to complete this invention.

[0023] (1) As a start ingredient for acquiring the gene of Proteinase A and proteinase-B gene this invention, 48180 shares of Candida BOIJINI ATCC is illustrated. Setting to this invention, a cloning process is a well-known approach (Molecular Cloning (1989) and Methods in Enzymology 194 (1991)). It can carry out by following. That is, the vector for transgenics incorporating the DNA fragment originating in all DNA of the (a) above-mentioned yeast or the cDNA fragment compounded from mRNA of the above-mentioned yeast is introduced into a host, and the gene library of the above-mentioned yeast is produced. (b) Subsequently, a desired clone can be chosen from this gene library, and the above-mentioned cloning process can be carried out by amplifying the clone concerned.

[0024] (a) After the extract of all DNA of the preparation yeast of the gene library of yeast prepares the protoplast of yeast and usually removes a well-known DNA extraction method, i.e., a cell residue, from the protoplast concerned, it can carry out alcoholic precipitate of the DNA under high salt concentration, and can be further performed using the approach of carrying out alcoholic precipitate and refining after a phenol or a chloroform extraction. In addition, although DNA can be extracted by the cell crushing method by a glass bead etc. besides the above-mentioned method of preparing a protoplast beforehand, it is desirable to perform the describing [above] protoplast method from the point that it is easy to prepare DNA of the amount of macromolecules. After digesting the obtained chromosome DNA with a suitable restriction enzyme and connecting with a suitable vector, a genomic library can be obtained by carrying out a transformation to a suitable Escherichia coli host.

[0025] Under the present circumstances, the available plasmid generally [the pBR system usually known as a well-known vector for gene library preparation as a vector used, a pUC system, a blue script (Bluescript) system, etc.] marketed can also be used. Moreover, a phage vector or cosmid of gt system or an EMBL system etc. can be used widely. The host who performs a transformation or transduction by the prepared vector for gene library production can adopt the thing according to the class of the above-mentioned vector.

[0026] (b) Using an indicator probe including an array peculiar to each gene, the clone which has desired Proteinase A and a desired proteinase-B gene can be chosen by the colony hybridization method, a plaque hybridization method, etc., and can be acquired from the selection above-mentioned gene library of a clone.

[0027] An array peculiar to Proteinase A and the proteinase-B gene which are used for a probe designs a primer for the codon usage of Candida BOIJINI to reference, amplifies specifically the DNA fragment for which it asks by the PCR method which uses the chromosome DNA of Candida BOIJINI as mold, and is acquired from the amino acid sequence saved by the protease of the various origins. Moreover, it is also possible to amplify specifically the DNA fragment for which it asks, and to acquire it by the PCR method which compounds 2 sets of oligonucleotides corresponding to the amino acid sequence of this protease refined from Candida BOIJINI, and uses the chromosome DNA of Candida BOIJINI as mold by making them into a primer. In addition, the compound oligonucleotide can also be used as a probe.

[0028] The decision and the check of the base sequence of a desired gene which are acquired by the above-mentioned approach can be performed by the chemistry changing method (Maxam-Gilbert, Methods in Enzymology, 65, and 499 (1980)) of for example, Maxam Gilbert, the dideoxy nucleotide chain ending method (Messing, J., Vieira and J., Gene, 19, and 269), its automated strange method, etc.

[0029] (2) The DNA array which carries out the code of the construction protease of a protease gene disruption plasmid is changed, and it is inserted into a suitable vector with DNA, a selective marker gene, etc. it is made to have functional protease protein produced, and is used as a gene disruption plasmid. It is producible by permuting this gene on a chromosome with the site specific inclusion using this plasmid. With the DNA array changed so that the functional protease protein used here could not be produced Carry out deletion of that by which the base of the DNA array which carries out the code of the protein was permuted, and the part, or at least one nucleotide is inserted (or addition). When the function of the product obtained even if the reading frame of the DNA array which carries out the code of the protein shifts, and these alterations are not discovered or it is discovered varies, it stops being able to carry out the code of the protein which has original protease activity. Such a changed DNA array is preferably producible by inserting a transformation marker

gene etc. during the DNA array which carries out the code of the protein. There is an advantage that the variant which has the protease gene changed considering the transformation marker gene introduced while being able to destroy the gene on a chromosome using such a DNA fragment as an index can be screened. As a selective marker gene used here, the gene which carries out the complementation of the auxotroph of the host of the antibiotic resistance gene of G418 grade, URA3, and LEU2 grade is illustrated. Inserting the constituent of an expression vector in a vector can be easily carried out by this contractor with the technique of common use with reference to the publication of the after-mentioned example.

[0030] (3) The *Candida BOIJINI* stock with which the acquisition protease activity of a protease deletion *Candida BOIJINI* stock was controlled as compared with the wild type strain is producible by carrying out the transformation of the suitable yeast host using the DNA array changed so that above-mentioned functional protease protein could not be produced, and replacing the gene of internality. There is the approach of removing the target gene on a chromosome physically and permuting by the alteration gene by gene substitution, as an approach for this. This is attained by carrying out the transformation of the yeast host using the straight chain-like DNA fragment which has a field by the side of 5 'the side and 3' of a target gene, and a homonous terminal sequence, and the alteration DNA fragment which contains preferably the gene divided by insertion of a transformation marker gene etc. URA3 changed gene which may be removed from a chromosome by spontaneous recombination desirable as this transformation marker gene can be used. The DNA array [homologous / side / that / 5 'side and 3' / as this URA3 changed gene] is made into the structure arranged in the same direction. Thereby, after being incorporated on a yeast chromosome, it becomes URA3 gene is able for the spontaneous recombination between this reiterative sequence to arise, and to fall out, and possible to reuse URA3 gene as a transforming gene marker. Under the present circumstances, since a Ura-stock serves as 5-fluoro-orotic acid (5-FOA) resistance, selection of the Ura-stock which fell out by homologous recombination with URA3 spontaneous gene out of the Ura+ stock of 5-FOA susceptibility is easy.

[0031] Moreover, the approach (Rothstein R., *Methods Enzymol.*, 194, and 281 (1991)) called pop in pop out as other approaches may be used. This is the approach of choosing the stock with which the functional gene was removed by the spontaneous homologous recombination which occurs between two genes which consist of a part of target genes of the internality produced after the transformation, and a part of alteration genes used for the transformation, and the changed gene was left behind after introducing into a target locus the plasmid DNA which contains an alteration gene by homologous recombination. By using URA3 gene as a marker also in this choice method, selection of the Ura-stock which fell out by homologous recombination with URA3 spontaneous gene out of the Ura+ stock of 5-FOA susceptibility is easy.

[0032] As an approach for carrying out the transformation of *Candida BOIJINI*, the protoplast method, an acetic-acid lithium, an electric pulse method, etc. can be used. It is ATCC although not restricted especially about the *Candida BOIJINI* stock used for a transformation. 48180 shares and IFO 10035 etc. shares etc. are illustrated. Furthermore, preferably, at least one auxotroph marker gene is this stock that carried out deletion, and a URA3 deletion stock, a LEU2 deletion stock, etc. are illustrated.

[0033] (4) The manifestation heterologous gene of a heterologous gene is performed by introducing into a suitable host cell the heterologous gene expression vector inserted into the suitable vector with the manifestation unit which has the structural gene of a promoter array and different-species protein, and a terminator array in the direction of the reading frame of an imprint, the selective marker gene, etc. When using a protease deletion mold *Candida BOIJINI* stock, a prosthetic device gene is destroyed as mentioned above, and a transformation is carried out by DNA which carries out the code of the different-species protein next. Or it is also possible to acquire a protease deficit stock afterwards for the stock by which the transformation was carried out by the heterologous gene expression vector already made into the object as mentioned above. It is also possible to carry out a transformation to a heterologous gene expression vector simultaneously furthermore with the protease gene with which the above was changed.

[0034] As a promoter for a recombination different-species protein manifestation, the promoter (JP,5-344895,A) of the alcohol oxidase gene of *Candida BOIJINI*, the promoter (the [international disclosure] WO 97/No. 10345) of a formic-acid dehydrogenase gene, etc. are illustrated. As a terminator, the terminator (JP,5-344895,A) of the alcohol oxidase gene of *Candida BOIJINI*, the terminator of a formic-acid dehydrogenase gene, the terminator (the [international disclosure] WO 97/No. 10345) of an actin gene, etc. are illustrated. In addition, secretion of different-species protein is attained by resembling a different-species protein amino terminal and connecting the signal sequence for secretion. The secretion transit peptide array of alpha factor of baker's yeast (*S.cerevisiae*) besides the secretion transit peptide array of the proteinase A offered by this invention as a signal sequence for such secretion etc. can be used.

[0035] An expression vector is made to include in the host chromosome DNA, or is made to exist in the state of a plasmid using the vector which has the autonomy duplicate array in which self-renewal is possible by host intracellular. As for the copy number of the heterologous gene which exists in host intracellular, one copy may also be plural. Thus, the gene expression product made into the object is acquirable by cultivating the obtained transformant and refining it from the culture obtained.

[0036] As a culture medium, mineral, such as a phosphoric acid, sodium, a potassium, magnesium, calcium, iron, copper, manganese, and cobalt, is added to one or more sorts of nitrogen sources, such as one or more sorts of carbon sources, such as a methanol, glycerol, and a glucose, and a yeast extract, trypton, a meat extract, a peptone, casamino acids, and ammonium salt, and what carried out expedient addition of the micronutrient, such as various vitamins, amino acid, and a nucleotide, if needed further is mentioned to them.

[0037] pH of a culture medium has the desirable range of 5-8. Moreover, 15-37 degrees C of culture temperature are usually around 28 degrees C preferably. Culture time amount is about 24 - 1000 hours, and culture can be carried out by the batch culture or continuous culture under standing, a shaking, stirring, and aeration. After culture termination, in order to extract a gene product from this culture, the usual protein purification means can be used. For example, when produced in a transformed cell, the crude protein solution in which a biomass is included with a conventional method and it includes a gene product by sonication, grinding processing, application-of-pressure crushing, etc. is acquired. A protease inhibitor is added if needed. Gene products can be collected from the culture medium itself when produced in a culture supernatant. Filtration, centrifugal separation, etc. remove a solid part for the obtained solution, and a crude-protein solution is obtained. The nucleic acid by protamine processing etc. is removed as occasion demands.

[0038] Separation purification of the object protein can be carried out by combining the purification technique, such as a salting-out method, solvent settling, dialysis, ultrafiltration, gel electrophoresis or ion exchange chromatography, gel filtration chromatography, reversed phase chromatography, and an affinity chromatography, from a crude-protein solution.

[0039]

[Example] This invention is not limited by these, although an example is given in order to explain this invention still more concretely.

<Example 1> *Candida* Cloning *Candida* of the proteinase A gene (PEP4) of *boidinii boidinii* ATCC Acquisition of PEP4 gene and its base sequence determination were performed from 48180 shares.

[0040] (1-1) production baker's yeast (*Saccharomyces cerevisiae*) of a probe (Woolford, C.A. et al., *Mol. Cell. Biol.* 6, and 2500-2510 (1986)) And *Pichia pastoris* (*Pichia pastoris*) The oligonucleotide of the base sequence corresponding to amino acid sequence (single-character notation): DFAEATSEPL and PYDYTLEVSGSCI which are saved by the proteinase A of the origin (Patent Publication Heisei No. 506117 [six to] official report) *Candida* The codon usage of *boidinii* is taken into consideration. As follows It compounded :P RA5: 5'-GATTTTGCWGAAGCWACWTCWGAACCGGTTT-3'; and PRA3 : 5'-ATACAWGAWACTTCYAAWGTRTAATCCTAWGG-3'.

[0041] A primer PRA5 corresponds to amino acid sequence DFAEATSEPL, and a primer PRA3 is the array of the complementary strand of the base sequence corresponding to amino acid sequence DFAEATSEPL. *Candida* cultivated by the YPD culture medium (1% [of yeast extracts], peptone 2%, and glucose 2%, pH6.0) *boidinii* From the biomass of 48180 shares of ATCC(s), Chromosome DNA was prepared by the potassium acetate method (Methods Enzymol., 65, and 404 (1980)).

[0042] *Candida* Primers PRA5 and PRA3 are mixed with the *boidinii* chromosome DNA, and it is Ex. PCR (it is [degrees C / 94] 2 minutes at 1 minute and 72 degrees C in 30 seconds and 50 degrees C) (x30 cycle) using Taq polymerase (TAKARA SHUZO CO., LTD.) was performed. The DNA fragments of about 0.6 amplified kbs are collected, and it is pT7. Blue Cloning was carried out to T-Vector (nova JIEN). It is *Saccharomyces* when the base sequence of the insertion DNA fragment of the obtained plasmid was determined using Dye primer cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.). *cerevisiae* And *Pichia* Since the base sequence which carries out the code of the amino acid sequence of PEP4 gene of the *pastoris* origin and the amino acid sequence with high homology was accepted, it is *Candida* about this DNA fragment. It was concluded that it was a part of PEP4 gene of *boidinii*. 0. The insertion DNA fragment of 6kb cut the plasmid by SalI and EcoRI, and collected them after agarose electrophoresis.

[0043] (1-2) Production of a library, and screening *Candida boidinii* Various restriction enzymes cut the chromosome DNA of 48180 shares of ATCC(s), and agarose gel electrophoresis was performed 0.8%. It is Hybond about separated DNA. N+ nylon membrane (Amersham) transfer was carried out. The radioactive indicator of the DNA fragment obtained in the example (1-1) was carried out using the megger primer DNA labeling system (Amersham), and Southern five RIDAZESHON was performed. Hybridization was performed according to the conventional method (Molecular cloning 2nd edn., ed. Sambrook, J., et al., Cold Spring Harbor Laboratory U.S.A., 1989). It was thought that PEP4 gene existed in a result and the EcoT22I fragment of about 5.5 kbs. Then, the library was produced that cloning of the DNA fragment should be carried out. *Candida* The chromosome DNA of *boidinii* was cut by EcoT22I, and the DNA fragments near 5.5kb were collected from gel after agarose electrophoresis. After carrying out ligation to pUC118 which cut the collected DNA fragment by PstI, the transformation was carried out to the *Escherichia coli* DH5alpha stock by the approach (Gene, 10, and 63 (1980)) of Hanahan, and the library was produced.

[0044] These libraries were screened by the colony hybridization which used the above-mentioned DNA fragment as the probe. The plasmid pCPRA1 and the insert selected the clone holding pCPRA2 which is hard flow from the obtained electropositive clones.

[0045] (1-3) The restriction enzyme map of the base-sequence-determination plasmid pCPRA1 was produced (drawing 1). As a result of various restriction enzymes' cutting a plasmid pCPRA1 and performing analysis by Southern hybridization, it was thought that PEP4 gene existed in the BglII-EcoT22 I region of about 3.5 kbs of drawing 1 . It is pBluescript about the HindIII fragment (field between HindIII (s) shown by the underline by drawing 1) of 1.7kb to the SmaI part of pUC18 in order to determine the base sequence of this field, after flush-end-izing the BglII-EcoRV fragment (field between BglII shown by the underline by drawing 1 , and EcoRV) of 2.2kb. II Cloning was carried out to the HindIII part of SK+ in both directions, respectively. The deletion mutant was acquired from each plasmid using double-stranded Nested Deletion Kit (Pharmacia Corp.). It is a base sequence Dye primer cycle sequencing FS Ready Reaction Kit And it determined using Dye terminator cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.). By connecting the acquired base sequence, the base sequence shown in the array number 1 was acquired.

[0046] It begins from the 1009th in the base sequence of the array number 1, and the open reading frame which consists of 1263 base pairs which finish as the 2271st exists in it. The amino acid sequence (array number 2) presumed from this open reading frame, and *Saccharomyces cerevisiae* And *Pichia pastoris* When homology with the proteinase A of the origin was questioned, 75% and 68% of amino acid was the same respectively. The transit peptide presumed by cutting [signal sequence] point prediction (von Heijne, *Nucleic Acids Res.*, 14, and 4683 (1986)) was 22 amino acid from a methionine to the 22nd alanine.

[0047] <Example 2> *Candida* Production *Candida* of the proteinase A gene (PEP4) destructive stock of *boidinii* PEP4 gene was destroyed by the transformation which used URA3 gene of *boidinii* as the marker. As a host, it is *Candida boidinii* Variant *Candida boidinii* of URA3 gene of 48180 shares of ATCC(s) 612 shares of SK was used. *Candida* 612 shares of *boidinii* SK(s) were acquired according to the well-known approach (Sakai Y. et al., *J. Bacteriol.*, 173, 7458 (1991)).

[0048] (2-1) As shown in production drawing 2 of a PEP4 gene-disruption vector, the plasmid pDPRA1 which permuted the SnaBI-EcoRV field of about 2 kbs of PEP4 gene by URA3 gene was produced. In order to acquire a uracil demand stock from a PEP4 gene-disruption stock again, based on Sakai's and others report (Sakai Y. et al., *J. Bacteriol.*, 174, and 7458 (1992)), URA3 gene which had a repeating structure before and after the structural gene was used as a marker.

[0049] *Candida* About the SalI-PstI fragment of 2.6kbs containing URA3 gene (Sakai Y. et al., *J. Ferment. Bioeng.*, 73, and 255 (1992)) of *boidinii*, it is pBluescript. II SalI of SK- and pCBU3 inserted between PstI parts were produced. pCBU3 is cut by SalI and it is T four. After carrying out flush end processing by DNA polymerase, it cut by XbaI further and the DNA fragment containing 5' side of URA3 gene of 0.9kb was isolated. Moreover, pCBU3 is cut by PstI and it is T four. After carrying out flush end processing by DNA polymerase, the DNA fragment of 2.6kbs further cut and obtained by KpnI and the DNA fragment of the above-mentioned 0.9kbs were inserted in KpnI-XbaI of pUC19, and Plasmid pURP was obtained. Consequently, the reiterative sequence of about 0.9 kbs will exist in the DNA fragment of 3.5kbs obtained by carrying out SalI cutting of the pURP before and behind a URA triad gene (drawing 2).

[0050] pCPRA1 and pCPRA2 by which PEP4 gene was inserted in the reverse sense were cut by SnaBI and EcoRV, and the XhoI linker (TAKARA SHUZO CO., LTD.) was inserted. The DNA fragment of 3.5kbs obtained by carrying out SalI cutting of the pURP to the XhoI part of the obtained plasmid was inserted, and the plasmid pDPRA1 was obtained (drawing 2).

[0051] (2-2) Cut pDPRA1 obtained by (2-1) of transformation this example by SalI, and it is *Candida boidinii* The transformation was performed to 612 shares of SK by the acetic-acid lithium method (Ito, H. et al., *J. Bacteriol.*, 153, and 163 (1983)). The PEP4 gene-disruption stock was screened by performing Southern analysis of the chromosome DNA about the obtained transformant. Namely, 612 shares of hosts SK and the chromosome DNA of the transformant were cut by SalI and NdeI, and Southern analysis was performed by using as a probe the DNA fragment of 1.7kbs obtained by carrying out SnaBI cutting of the pCPRA1 with SalI (drawing 3). Although a band is detected by 3.8kbs in 612 shares of hosts SK as shown in drawing 3 , a band is detected on a destructive stock in the location of 5.4kbs.

[0052] It is *Candida boidinii* about this destructive stock. *Candida boidinii* It was named 740 shares of SK. *Candida boidinii* After cultivating 740 shares of SK to a stationary phase by the YPD culture medium, the stock which shows resistance to 5-fluoro orotidine acid (5-FOA) was acquired. Acquisition of a 5-FOA resistant strain followed the approach given in an experiment document (the Ishida **** Ando **** / editing, a gene expression experiment manual, Kodansha SAIENTIFIKU, 1994). The stock with which URA3 gene was missing was screened by performing the same Southern analysis as the time of acquiring a PEP4 gene-disruption stock for the chromosome DNA of a 5-FOA resistant strain. On the stock as for which the band detected in 740 shares of SK in the location of 5.4kbs as shown in drawing 3 lacked URA3 gene, the band was detected in the location of 2.8kbs. It is *Candida boidinii* about the yeast with which URA3 gene was missing. *Candida boidinii* It was named 741 shares of SK, and international deposition was carried out on September 1, Heisei 10 in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, the Ministry of International Trade and Industry, (Ibaragi Prefecture, Tsukuba-shi) at the bottom of a swine plague treaty, and trust number FERMBP-6482 were given.

[0053] <Example 3> *Candida* Cloning *Candida* of the proteinase-B gene (PRB1) of *Candida boidinii* Acquisition of PRB1 gene and its base sequence determination were performed from 48180 shares of ATCC(s).

(3-1) The production *Saccharomyces* of a probe *Cerevisiae* () [Moehle,] [C.M.] et al. and Mol.Cell.Biol.7, 4390-4399 (1987) And *Pichia pastoris* (Patent Publication Heisei No. 506117 [six to] official report) The oligonucleotide of the base sequence corresponding to amino acid sequence (single-character notation): GNGHGHHCAGT and ATAVLSGTSMA which are saved by the proteinase B of the origin *Candida* : compounded as follows in consideration of the codon usage of *Candida boidinii* [0054] PRB5 : 5'-GGTAAYGGTCAYGGTACHCAYTGTGCHGGWAC-3'; and PRB3 : 5'-GCCATWGAWGATAGCWGATAAARACDGCWGTDGC-3'.

[0055] A primer PRB5 corresponds to amino acid sequence GNGHGHHCAGT, and a primer PRB3 is the array of the complementary strand of the base sequence corresponding to amino acid sequence ATAVLSGTSMA. *Candida boidinii* Primers PRB5 and PRB3 are mixed with the chromosome DNA of 48180 shares of ATCC(s), and it is Ex. PCR (it is [degrees C / 94] 2 minutes at 1 minute and 72 degrees C in 30 seconds and 50 degrees C) (x30 cycle) using Taq polymerase (TAKARA SHUZO CO., LTD.) was performed. The DNA fragments of about 0.5 amplified kbs are collected, and it is pT7Blue. Cloning was carried out to T-Vector (nova JIEN). It is *Saccharomyces* when the base sequence of the insertion DNA fragment of the obtained plasmid was determined using Dye primercycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.). *Cerevisiae* And *Pichia* Since the base sequence which carries out the code of the amino acid sequence of PRB1 gene of the *Pastoris* origin and the amino acid sequence with high homology was accepted, it is *Candida* about this DNA fragment. It was concluded that it was a part of PRB1 gene of *Candida boidinii*. 0. The insertion DNA fragment of 5kb cut the plasmid by *Sall* and *EcoRI*, and collected them after agarose electrophoresis.

[0056] (3-2) Production of a library, and screening *Candida boidinii* Various restriction enzymes cut the chromosome DNA of 48180 shares of ATCC(s), and agarose gel electrophoresis was performed 0.8%. It is Hybond about separated DNA. N+ nylon membrane (Amersham) transfer was carried out. The radioactive indicator of the obtaining-by (3-1) of this example DNA fragment was carried out using the megger primer DNA labeling system (Amersham), and Southern hybridization was performed. Consequently, it was shown that PRB1 gene exists in the *EcoRI*-*HindIII* fragment of about 5.5 kbs and the *BglII*-*EcoT22I* fragment of about 4.5 kbs. Next, the library was produced that cloning of the *EcoRI*-*HindIII* fragment of about 5.5 kbs and the *BglII*-*EcoT22I* fragment of about 4.5 kbs should be carried out. *Candida* The chromosome DNA of *Candida boidinii* was cut by *EcoRI* and *HindIII*, and the DNA fragments near 5.5kb were collected from gel after agarose electrophoresis. The collected DNA fragment was inserted between *EcoRI* of pUC19, and a *HindIII* part, and the *EcoRI*-*HindIII* plasmid library was produced. It is pBluescript about a *BglII*-*EcoT22I* fragment similarly. II The *BglII*-*EcoT22I* plasmid library inserted between *BamHI* of SK+ and a *PstI* part was produced.

[0057] Screening by the colony hybridization which used the above-mentioned probe for these libraries was performed. The clone which holds a *BglII*-*EcoT22I* plasmid library to pCPRB1 and pCPRB2 from an *EcoRI*-*HindIII* plasmid library was selected as an electropositive clone by autoradiography.

[0058] The restriction enzyme map of pCPRB1 and pCPRB2 was produced (drawing 4). The obtained clone is *Candida*. Checking that it is PRB1 gene of *Candida boidinii*, and *Candida* The base sequence of the *ClaI* field of about 0.7 kbs of the minimum DNA fragment which the probe hybridized by the genomic Southern analysis mentioned above for the purpose of presuming the location and direction of an open reading frame of PRB1 gene of *Candida boidinii* was determined. About the *ClaI* fragment of 0.7kbs acquired from pCPRB2, the decision of this base sequence is pBluescriptII. It carried out using the plasmid which inserted in SK+ and was produced. The amino acid sequence (array number 5) presumed from the acquired base sequence (array number 6), and *Saccharomyces cerevisiae* And *Pichia* When homology with the proteinase B of the *Pastoris* origin was questioned, 76% and 77% of amino acid was the same respectively. From this result, it is *Candida*. It was presumed that the opening *Ryding* frame of PRB1 gene of *Candida boidinii* exists in the field shown by the arrow head of drawing 4.

[0059] <Example 4> *Candida* Production *Candida* of the proteinase-B gene (PRB1) destructive stock of *Candida boidinii* PRB1 gene was destroyed by the transformation which used URA3 gene of *Candida boidinii* as the marker. *Candida* acquired in the example 2 as a host *Candida boidinii* 741 shares of SK was used.

(4-1) The plasmid pDPRB1 which permuted the *ClaI* field of about 0.7 kbs of production PRB1 gene of a PRB1 gene-disruption vector by URA3 gene was produced as follows.

[0060] The DNA fragment of about 2.0 kbs which cut pCPRB2 by *ClaI* and *EcoRI* and were obtained was inserted in the *ClaI*-*EcoRI* field of pCPRB1. The obtained plasmid was named pCPRBdelta*Cla*. pCPRBdelta*Cla* is cut by *ClaI* and it is T four. The *XhoI* linker was inserted after [which is depended on DNA polymerase] carrying out flush end processing. The DNA fragment of 3.5kbs obtained by (2-1) of an example 2 by carrying out *Sall* cutting of the pURP of a publication to the *XhoI* part of the obtained plasmid was inserted, and the plasmid pDPRB1 was obtained (drawing 5).

[0061] (4-2) Cut pDPRB1 obtained by (4-1) of transformation this example by *HincII* and *EcoRI*, and it is *Candida boidinii* The transformation was performed to 741 shares of SK by the acetic-acid lithium method. The PRB1 gene-disruption stock was screened by performing Southern analysis of the chromosome DNA about the obtained transformant. Namely, 741 shares of hosts SK and the chromosome DNA of the transformant were cut by *BglII* and *HindIII*, and Southern analysis was performed by using as a probe the DNA fragment of 1.3kbs obtained by carrying out *BglII* cutting of the pCPRB1 with *ClaI* (drawing 6). The band detected in 741 shares of hosts SK in the location of 3kbs as shown in drawing 6 is detected by 5.8kbs on a destructive stock.

[0062] It is *Candida* about this destructive stock. *Candida boidinii* It was named 774 shares of SK. *Candida boidinii* The 5-FOA resistant strain was acquired from 774 shares of SK, and the stock with which URA3 gene was missing was screened. Southern analysis performed screening. The band detected in 774 shares of SK in the location of 5.8kbs as shown in drawing 6 was detected in the location of 3.2kbs

on the stock which lacked URA3 gene. It is Candida about this yeast. boidinii It was named 775 shares of SK.

[0063] <Example 5> Candida obtained by (2-2) of the measurement example 2 of the protease activity of a protease deficit stock boidinii SK740 (pep4) Candida obtained by (4-2) of a stock and an example 4 boidinii SK774 (pep4, prb1) A stock and Candida boidinii The shown protease activity of 48180 shares of ATCC(s) was measured. Each stock was cultivated to the stationary phase at 30 degrees C by the 2ml YPD culture medium. They are 0.2ml 100mM(s) about the biomass which carried out the harvest. It suspended in the Tris-HCl buffer (pH 7.5), the 0.8g glass bead (0.425 to 0.6 mm, sigma company) was added, and actuation of ice-cooling for 1 minute was violently repeated 5 times after stirring for 1 minute. The at-long-intervals alignment of the biomass crushing liquid was carried out by 4 degrees C and 10000 revolutions for 10 minutes, and the supernatant liquid fraction was acquired as a cell-free extract. The protein concentration of a cell-free extract was measured using the protein assay kit (Bio-Rad).

[0064] The enzyme activity of a cell-free extract measured proteinase A activity and carboxypeptidase Y activity according to the total theory (Jones, E.W., Methods Enzymol., 194, and 428 (1991)) of Jones. That is, proteinase A activity is the cell-free extract of 25microl, and final concentration 100mM. Glycine-HCl It measured at 37 degrees C in the 1ml reaction mixture containing a buffer (pH 3.2) and 1% of acid denaturation hemoglobin. After sampling the reaction mixture of 200microl, respectively after 0 minutes, 10 minutes, 20 minutes, and 30 minutes, 1-N perchloric acid of 100microl was added, and the at-long-intervals alignment was carried out by 10000 revolutions for 10 minutes. 100micro of supernatant liquid I is sampled, and it is 0.5M of 50microl. NaOH was added and the isolation peptide content in this solution was measured using DC protein assay kit (Bio-Rad). Proteinase A activity defined the amount of enzymes which separates the peptide of 1microg in 1 minute as one unit. Although the proteinase A activity of 49.3 units was detected per 1mg of cell-free extracts in 48180 shares of ATCC(s), activity was not detected in 740 shares of SK, and 774 shares of SK.

[0065] After carboxypeptidase Y activity mixed the cell-free extract of 100microl, the buffer (100mM Tris-HCl (pH7.5), 1mM CaCl2) of 500microl, and the substrate solution (6mM N-benzoyl-L-thyrosin-p-nitroanilide dissolved by dimethylformamide (sigma company)) of 20microl and stirred them well, it reacted for 30 minutes at 37 degrees C. It is 1.5M of 600microl to this. The acetic acid was added, the reaction was suspended, it filtered with the 0.22-micrometer filter, and the absorbance in 405nm was measured. Carboxypeptidase Y activity defined the amount of enzymes which separates the para nitroaniline of 1nmol in 1 minute as one unit. The carboxypeptidase Y activity of each, 0.72 units, 0.28 units, and 0.05 units was detected per [which 48180 shares of ATCC(s), 740 shares of SK and 774 shares of SK show] 1mg of cell-free extracts, and it was checked that carboxypeptidase Y activity decreases substantially by the protease genetic defect.

[0066] <Example 6> Candida by which the secretory production protease gene of heterologous gene protein was destroyed By discovering a cow origin cathepsin C gene using a boidinii stock, it checked that the amount of secretion of cathepsin C increased. Moreover, it also checked functioning as a signal sequence for the pre array of PEP4 gene obtained by (1-3) of an example 1 making heterologous gene protein secrete.

[0067] (6-1) The Homo sapiens origin cathepsin C gene reported to cloning ** of a cow origin cathepsin C gene was acquired in PCR, and the obtained DNA fragment was used as a probe. :HCat-5 which compounded the following oligo oligonucleotides according to the base sequence (Patris, A.et al, FEBS Lett., 369, and 326 (1995)) of a Homo sapiens cathepsin C gene : 5'-CAAGGCTTTGAGATTGTGTTGAATGACTAC-3' and HCat-3 : 5'-TCTGAGATTGCTGCTGAAAGTCTACAGTCT-3'.

[0068] It is QUICK-Screen as template DNA. Human cDNA Library Panel (Clontech) was used. Primer HCat-5 and HCat-3 are mixed with template DNA, and it is Ex. PCR (it is [degrees C / 94] 2 minutes at 30 seconds and 72 degrees C in 30 seconds and 60 degrees C) (x30 cycle) using Taq polymerase (TAKARA SHUZO CO., LTD.) was performed. From the library of the placenta origin, the DNA fragments of about 1.2 amplified kbs are collected, and it is pT7. Blue Cloning was carried out to T-Vector (nova JIEN). Using Dye primer cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.), the base sequence was determined and it checked that the Homo sapiens origin cathepsin C gene was inserted. 1. The insertion DNA fragment of 2kb cut the plasmid by SmaI and XbaI, and after agarose electrophoresis, they were collected and was used for it as a probe DNA fragment.

[0069] Bovine purchased from Stratagene as a library for acquiring a cow cathepsin C gene Spleen The cDNA library was used. It screened by plaque hybridization from the recombination phage clone of about 1 million made to appear according to an attached protocol. In six obtained electropositive recombination phage and the helper phage of attachment to a library, it is Escherichia coli XL1-Blue. You made it infected with an MRF⁺ stock, it cultivated at 37 degrees C for 3 hours, and pBluescript which has an object cDNA fragment was started. The digestive liquor which processed culture medium for 20 minutes at 70 degrees C was infected with the Escherichia coli SOLRTM stock, and ampicillin resistance selected the Escherichia coli which has recombination plasmid DNA.

[0070] As a result of determining the base sequence by the side of a five prime end and a three-dash terminal for six recombination plasmids using Dye primer cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.), pBC 20-2 was selected as a clone which has the longest cDNA fragment. The base sequence of the insertion DNA fragment of pBC 20-2 was determined using Dye terminator cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.), and the base sequence shown in the array number 7 was acquired. The amino acid sequence shown in the array number 8 from the acquired base sequence was acquired. When this array was compared with the amino acid sequence of Homo sapiens cathepsin C, 89% of amino acid was the same. The amino terminal of a pro field was considered to be the 226th leucine of the array number 8 for the amino terminal of the 20th aspartic acid of the array number 8, and a mature field. The array which carries out the code of the initiation methionine was not included in pBC 20-2, but it was thought that a part of pro field was missing.

[0071] (6-2) It is Candida about the cow cathepsin C obtained by (6-1) of construction this example of a cow cathepsin C manifestation plasmid. In order to carry out a secretion manifestation using boidinii, the pre field (array number 4) of the proteinase A obtained by (1-3) of an example 1 was connected with the amino terminal side of the pro field-mature field of cow cathepsin C. Moreover, the base sequence of the pro field-mature field of cow cathepsin C is Candida. It changed into the base sequence array using a codon with high operating frequency in boidinii. It designed so that a NotI recognition site might furthermore be formed in 5'3 of upstream and translation termination codon (TAA)' downstream of the translation initiation codon (ATG) of a structural gene (array number 9). Designed DNA is the approach shown in drawing 7, and was compounded using PCR : which shows the base sequence of each primer in drawing 7 below -- A1F:5'-

GTACATATCCAGATCTATTAGGTACTTGGGTCTTTCAAGTTGGTTCTTCTGGTTCACAAAGAGATGTTAATTGTTCTGTTATGGGTCCTCC-3';

A1R:5'-

GCAAAACCATTATAATCATTCAAGACAATTTGAAACCTTGATTATAGATAATAGTGAAATGACCAGAATTACCAAAATCATCATAAGCA

-3';
 A2F:5'-
 GGGGGGCGGCCGCATGAAGTTCACAATTCCTTTTTCTGTGCTTTCTCTATCTTAGCTGCTACTACTTTAGTTGATGCTGATACTCCAGC
 -3';
 A2R:5'-
 CCCCCACTAGTCCTAGGACATCATGAACCCAACCTGTCATAGTTTCATGACAATAAGAAGTAACCTTTACCACCTTCTTCTTTATATTTAA/
 -3';
 B1F:5'-
 CGTTAATACTGCTAGATTAGCTGGTTTAGAAGAAACATACTCTAATAGATTATATCGTTATAATCATGATTTCTGTCAAAGCTATTAATGCT
 -3';
 B1R:5'-
 TACGAGAATGACCACCACCTCTTCTAATCATTTCTTTAAGAGTTAATGTTTCATATTCCATATAAGGAGCAGCAGTCCAAGATTTTTGAA/
 -3';
 B2F:5'-
 GGGGGGCGGCCGCGGGGCTAGGTAGAAATTGGGCTTGTTTCACTGGTAGAAAGACTGGTAATACTTCTGAAAATGTTAACGTTAATAC
 -3';
 B2R:5'-
 CCCCCACTAGTAGGTAAGTGAAGATTTTCTTCTGAATTTCAAGCAGTAATAGGTGCAGGTTTAGGTCTAGGTATTCTACGAGAATGACCA
 -3';
 C1F:5'-
 TTGCTTCTATGGGTATGATGGAAGCTAGAATTAGAATTTTGACTAATAATACTCAAACCTCCTATCTTATCTCCACAAGAAGTTGTCTCTTC
 -3';
 C1R:5'-
 ATGGAGAATCAGTACCAGTATATGGAAAACAATCTTCTTCAACTAGACCAAAGTCCTGAGCATATTTACCAGCAATTAAGTATGGGAAAC
 -3';
 C2F:5'-
 GGGGGACTAGTTGGGATTGGAGAAATGTTTCATGGTATTAACCTTTGTTACTCCTGTTAGAAATCAAGGTTTCATGTGGTTCTTGTTACTCAT/
 -3';
 C2R:5'-
 CCCCCAAGCTTCATTACAACCACCATAGAAACCACCAACATAATGATATTCAGAAGAGTAATATCTGAAACAACCTTCTTTCAATCTACA
 -3';
 D1F:5'-
 ATTATAGAAAAGGTGTTTATCATCACACTGGTTTAAAGAGATCCATTTAATCCATTTGAGCTCACTAATCATGCTGTCTTATTAGTTGGTTA
 -3';
 D1R:5'-
 GTACCTCTTCTAATTCTAAAGTAACCATTTTACCCCCAAGAAGTACCCCATGAGTTCTTAACAATCCAATAATCTAAACCAGAAGCAGCA
 -3';
 D2F: 5'-
 GGGGGAAGCTTTGATGAAATTAGAATTAGTTCATCAAGGTCCTATGGCTGTTGCTTTGAAGTCTATGATGATTTCTTACATTATAGAAA/
 -3'; and D2R:5'-
 CCCCCCTCGAGGCGGCCGCTTATAATTTAGGAATAGGAGTAGCAGCTAAAGCAATAGATTCAATAGCACATTCATCAGTACCTCTTCTA/
 -3'.

[0072] Field A mixes primer A1F and A1R first, and is Ex. PCR (it is [degrees C / 94] 30 seconds at 1 minute and 72 degrees C in 30 seconds and 60 degrees C) (x20 cycle) using Taq polymerase (TAKARA SHUZO CO., LTD.) was performed, and the double stranded DNA was compounded. A phenol/chloroform extraction, and ethanol precipitate were performed after reaction termination, and it dissolved in TE buffer of 1/2 capacity (25microl) of PCR reaction mixture. This 2micro [of solutions I], primer A2F, and A2R is mixed, and it is Ex. PCR (it is [degrees C / 94] 30 seconds at 1 minute and 72 degrees C in 30 seconds and 60 degrees C) (x20 cycle) using Taq polymerase (TAKARA SHUZO CO., LTD.) was performed. pBluescript after collecting the amplified DNA fragments and cutting by NotI and SpeI II It inserted between NotI-SpeI of KS+. The time Field A was compounded correctly was checked using Dye primer cycle sequencing FS Ready Reaction Kit (PerkinElmer, Inc.). The obtained plasmid was named pCT-A.

[0073] It compounds by the same approach as Field A using the primer shown in drawing 7 also about Fields B, C, and D, and is pBluescript, respectively. II Plasmid pCT-B, pCT-C, and pCT-D inserted in KS+ were obtained. Plasmid pCT-AB which inserted the NotI-StyI fragment cut down from pCT-A between NotI-StyI of pCT-B, and plasmid pCT-CD which inserted the SpeI-HindIII fragment cut down from pCT-C between SpeI-HindIII of pCT-D were produced. The SpeI-XhoI fragment cut down from pCT-CD was inserted between SpeI-XhoI of pCT-AB, and plasmid pCTC-S1 was produced.

[0074] Candida started from pCBU3 boidinii Candida started from the SalI-PstI fragment and pFdhPT (WO 97/10345) of 2.6kbs containing a URA gene boidinii The KpnI-EcoT22I fragment of 2.1kbs including a formic-acid dehydrogenase gene promotor / terminator field was inserted between KpnI-SalI of pUC19, and the marker gene produced the heterologous gene manifestation plasmid pFexU3 by the formic-acid dehydrogenase gene promotor / terminator with URA3 gene (drawing 8). The NotI fragment containing the cathepsin C gene started from pCTC-S1 was inserted in the NotI part of pFexU3, and cow cathepsin C manifestation plasmid pECTC-S1 was produced (drawing 8).

[0075] (6-3) Cut plasmid pECTC-S1 obtained by (6-2) of transformation this example by BamHI, and it is Candida. boidinii The transformation was carried out to 612 shares of SK, and 741 shares of SK. Ten colonies per each host stock of the obtained transformant were gathered, and the cathepsin C activity secreted in a culture medium was measured. First, shaking culture was carried out at 30 degrees C for 48 hours in the GLYS culture medium (culture medium of pH5.5 containing glycerol 3%, Yeast Nitrogen Base 0.67%, and Yeast Extract 0.5%). 3000 revolutions and the biomass which carried out the harvest by centrifugal [for 5 minutes] were suspended in a GLYS culture medium and equivalent MYS (culture medium of pH5.5 which contains Yeast Extract0.5% methanol 1.5% and Yeast Nitrogen Base 0.67%), and shaking culture was carried out at 30 more degrees C for 48 hours. 3000 revolutions and the culture

supernatant acquired by centrifugal [for 5 minutes] were condensed 50 times after culture using the microcomputer -30 (Amicon), and cathepsin C activity was measured by the approach shown below.

[0076] After mixing with the concentration culture supernatant of 2microl the buffer (a 50mM citric-acid-sodium-citrate buffer (pH5.0), 10mM NaCl, 1mM beta-mercaptoethanol, and 4mM(s) Glycyl-L-phenylalanine-p-nitroanilide of a substrate (what was dissolved in 200mM (s) is diluted with a sigma company and dimethylformamide)) of 200microl, it was left at 37 degrees C for 2 to 10 hours, and the absorbance in 405nm was measured. 16, 8, 4 and 2 of the cow cathepsin C purchased from BERINGA as a reference standard, and 1 or 0.5microg [/ml] solution were prepared, and the cathepsin C activity of each sample was computed from the standard curve which produced this as a sample. The cathepsin C volume which each transformant showed is shown in drawing 9 . Candida by which the proteinase gene was destroyed as shown in drawing 9 The direction at the time of using a boidinii stock as a host was excellent in cathepsin C productivity.

[0077]

[Effect of the Invention] Candida to which protease (or proteolysis) activity decreased by this invention A boidinii stock is offered and the yield of this protein can be raised by preventing disassembly of object protein in the manifestation system which made this yeast the host.

[0078]

[Layout Table]

SEQUENCE LISTING <110> Kirin-Brewery-Company, Limited <120> Candida boidinii-strains-and use-thereof-as-hosts for-preparing-heterologous proteins <130> P98-0444 <160> 9<210> 1<211> 3486<212> DNA<213> Candida boidinii <400> 1agatcttggg atacgcattc ttccgccaac caccaaccac cagccttcca gcaactagcc60agcagccagc agcaggccca aagatgtggc accggcatga aacaatggct gctggcgcg120aaacaactgc ggcagggtca catctcccat tgttttccac gcgctgttgc tgcattgggc 180cttgtgagaa atacaaatag ggaaagcgat acatacgtaa tgtatgcaat gtatgtaag 240tatgcaatta caattgttgc cctctctctt tctacggctc ttctatggc tcttctctct 300gtatgaacc cactggccct atctctgtcc tctgtgtctc tatctccatc ttccctcttt 360cctcttctct ctgtcttctt ctatctaca catatcactc ttattctctt tgcctgttg 420ctcatccct gaactgtgcc ctctctccc tctctcttcc tctctcacc ttagtattgt 480cttggcccaa tgcaattct aactccattt gcaatcacat tcacatttcc tctccattca 540actcttcac tttgtctctc ttatcaatta attgattaat caatccacct cctctctatc 600tttactctc toccattacc acatcttctt atcagtctgt ctccatcacc ttctccatca 660aggccattat aaattaacgc ccaacaccat tgccatccat cccccattat catacaacta 720aagagtattc taatcaatcc atctccgttt gtcatctgtc tcaaatatca cacaagctaa 780tcaattocct taaagaatta atcctottaa ttgtattgat agtcatttag cattcaccaa 840aatttgataa gtatagaatc taagtataaa aatataaaa agaacttttc tcttcaaac 900atttaaccgc ccatttccct aaaattaag gtatataat tacacaaatt caaccattaa 960aaggaaaaaa aaagaaaaaa actacttctc aaaaaagaatcttgcgaat gaagtcaca 1020atccctttt ctgtcgtttt cagatcttta gctgctacta ccttagttga tgccaagtt 1080cactcaattc caattaaaaa acactctta gaagaaactt taaagatat ttcttataat 1140gattatttag cttctttaa gaataaatat atctcattat ataacaagca tcaactaaa 1200aacgcgggtg aatctattga aggtgatcaa caacaccctt ttatccatt cgttgaagt 1260gtcagtggtg aattcaaaga ttcaaaaact gatgctcctt taactaacta tatgaatgct 1320caatatttca cagaaattca attaggtacc ccagggtcaag tctttaaagt tatcttagat 1380accggttctt ccaatttatg ggtcccagggt aaggattgtt cttctttagc ttgttactta 1440cactcaaagt atgatcaga tgaatcgtca acttataaga aaaacgttac cgaatttgc 1500attagatag gtactgttgc tttagaaggt tttgtctctt ctgatacttt aaccattgga 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1.This document has been translated by computer. So the translation may not reflect the original precisely.

2.*** shows the word which can not be translated.

3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] This drawing shows the restriction enzyme map of the plasmid pCPRA1 containing a proteinase A gene.

[Drawing 2] This drawing shows the construction procedure of the proteinase A gene disruption plasmid pDPRA1.

[Drawing 3] This drawing is *Candida. boidinii* The restriction enzyme map of PEP4 locus of 612 shares of SK, 740 shares of SK, and 741 shares of SK is shown.

[Drawing 4] This drawing shows a proteinase-B gene and the restriction enzyme map of plasmids pCPRB1 and pCPRB2.

[Drawing 5] This drawing shows the structure of the proteinase-B gene disruption plasmid pDPRB1.

[Drawing 6] This drawing is *Candida. boidinii* The restriction enzyme map of PRB1 locus of 741 shares of SK, 774 shares of SK, and SK775 is shown.

[Drawing 7] This drawing shows the construction procedure of plasmid pCTC-S1.

[Drawing 8] This drawing shows the construction procedure of plasmid pECTC-S1.

[Drawing 9] This drawing is *Candida. boidinii* The cathepsin C activity of the culture-medium supernatant liquid of the cathepsin C manifestation stock which made the host 612 shares of SK and 741 shares of SK is shown.

[Translation done.]

NOTICES *

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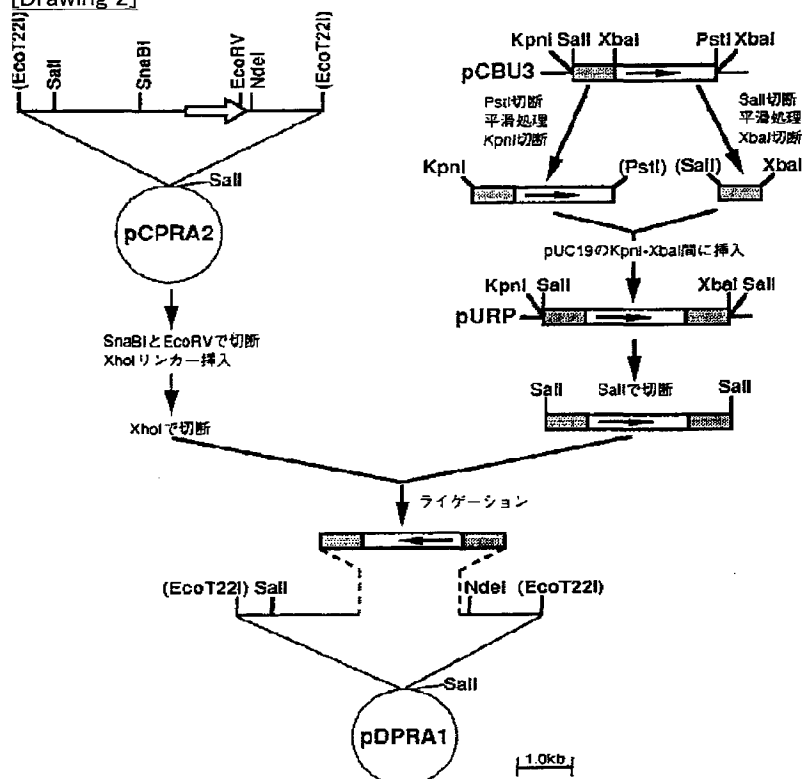
- 1.This document has been translated by computer. So the translation may not reflect the original precisely.
- 2.*** shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

DRAWINGS

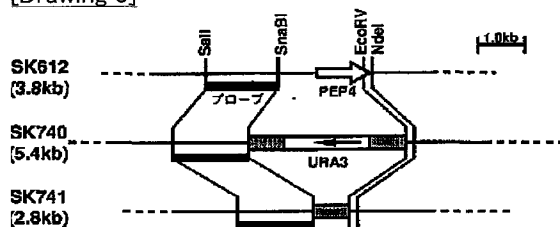
[Drawing 1]



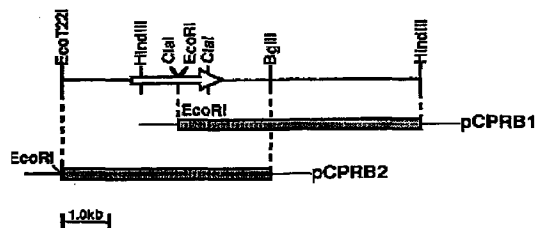
[Drawing 2]



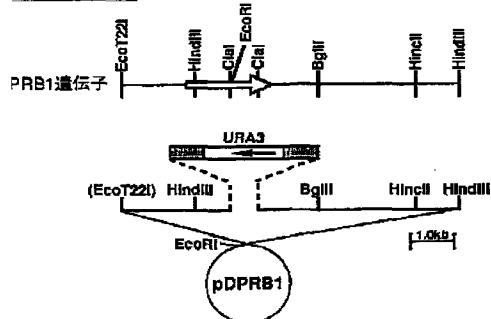
[Drawing 3]



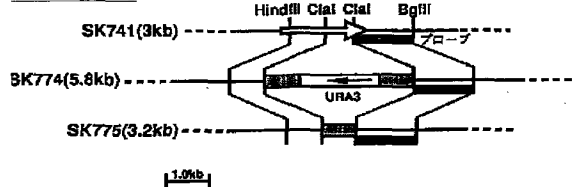
[Drawing 4]



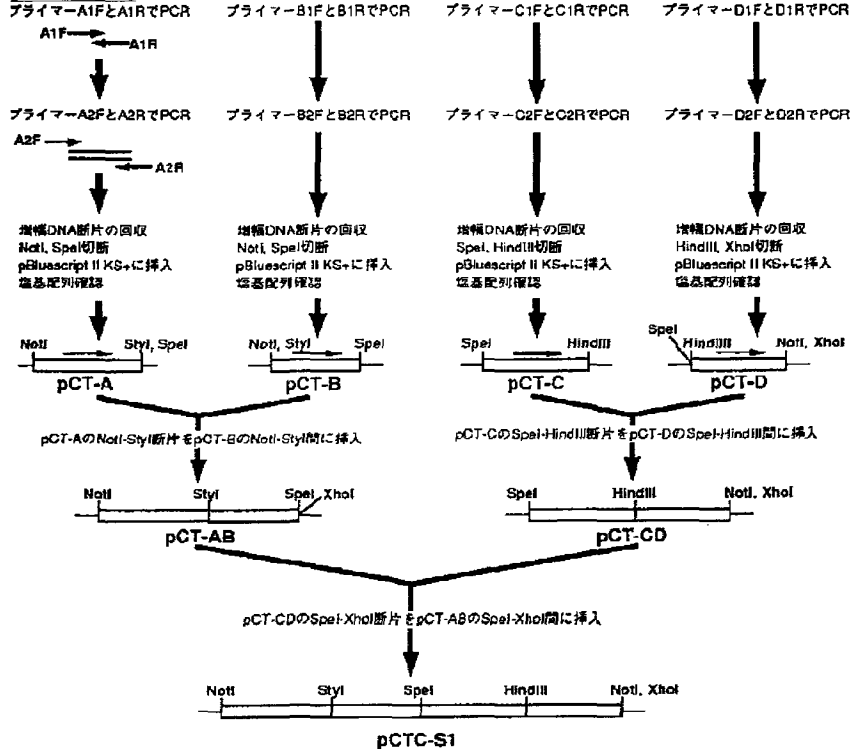
[Drawing 5]



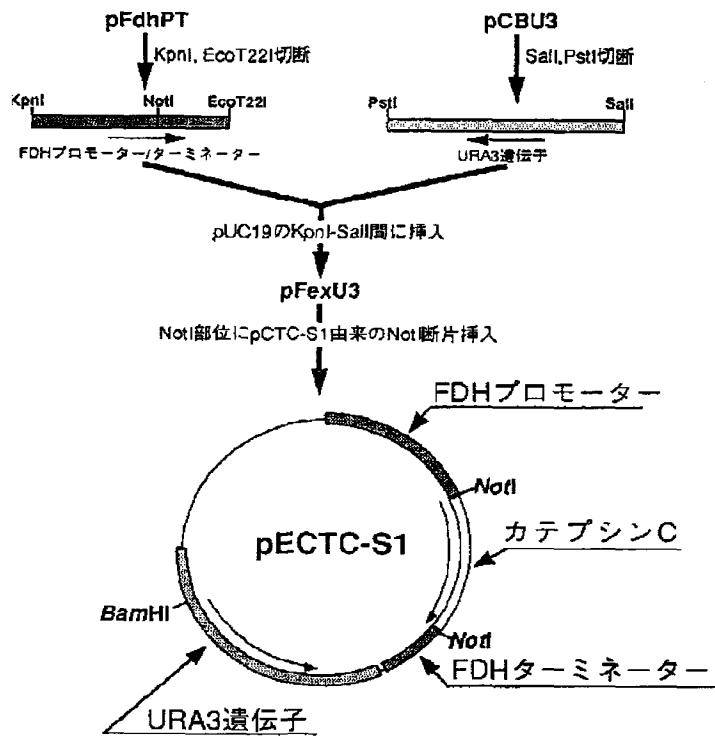
[Drawing 6]



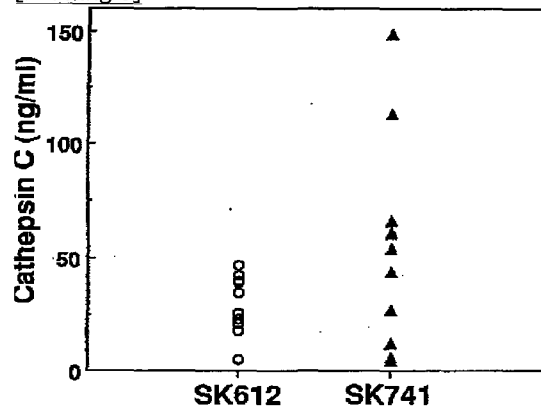
[Drawing 7]



[Drawing 8]



[Drawing 9]



[Translation done.]